THE EFFECT OF AUTOLOGOUS TUMOUR VACCINE ON BENZO-A-PYRENE INDUCED SQUAMOUS CELL CARCINOMA OF SKIN IN MICE

BY

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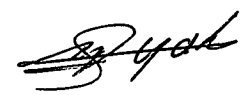

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STATEMENT OF ORIGINALITY

The work presented in this thesis was performed in the Department of Surgery, University Of Tasmania. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge contains no copy or paraphrase of material previously published by any other person except where due reference is made in the text.

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PROJECT AIMS

Dendritic cells (DC, an antigen presenting cell-APC) have been shown to be present in some tumours, and evidence indicates that these may be an indication to the ability of these tumours to provoke an immune response. This project investigated the effect of autologous tumour vaccine on benzo-a-pyrene-induced squamous cell carcinoma in mice, a tumour which is characterised by high density of DC within the tumour tissue. The aims of this thesis is to determine if tumours with high level of DC infiltration tend to respond to vaccine induced immunity, and to examine if DC can be used as a marker to reflect tumour response to vaccination correlating with appearance of tumour necrosis and appearance of non-DC leukocytes infiltration.

SUMMARY

Many studies have correlated the presence of infiltrating immune cells within solid tumours, with a more favourable prognosis. These observations indicate that the immune system may act to defend against tumour formation or growth. The generation of an immune response requires that the tumour cells express antigens recognised by the host immune system. The association between an impaired immune system with advanced tumours and poor prognosis suggests that appropriate stimulation of the immune system against tumour tissues might be beneficial.

Active specific immunotherapy with autologous tumour cells or extract has been shown to be effective in causing regression of tumour. The prerequisite for effective immunotherapy is the expression of structures on tumour cells which are antigenic to the immune system and may function as rejection antigens. Various attempts have been made to identify such antigens in different tumours. Many antigens have been detected in different tumours, some of these antigens are immunogenic and some are not.

In an attempt to overcoming the difficulty in identifying the tumour antigens and in determining which are immunogenic, could be used as an indicator of the immunogenicity of tumour cells. DC are an antigen presenting

cells, the presence of these cells in the region of tumour tissues may indicate the presence of an immunogenic antigens on tumour cells. In this study, squamous tumours induced by BP were chosen for immunotherapy by autologous tumour vaccine because these tumours were reported to have high numbers of dendritic. The application of tumour vaccine to mice possessing these tumours was shown to be effective and to lead to an enhanced immune response against tumour cells compared to controls. DC infiltration of tumours in test group was significantly higher than DC infiltration of control group, this correlating with high degree of tumour necrosis and infiltration of tumours by non-DC leukocytes in test group compared to the control group.

ABBREVIATIONS

APC	Antigen Presenting Cell
ALL	Acute Lymphocytic Leukemia
BCG	Bacillus Calmette Guerin
BP	Benzo-a-Pyrene
DC	Dendritic cell
DMBA	1,7 Dimethyl Benzo-a-Anthracene
DMSO	DiMethylSulphOxide
DNP	Dinitrophenyl
DTH	Delayed Type Hypersensitivity
C *Parvum*	Corynebacterium *parvum*
CSF	Colony Stimulating Factor
CTL	Cytotoxic T-Lymphocyte
CY	Cyclophosphamide
EBV	Epistien Bar Virus
Fc	Fc portion of the immunoglobulin
FcR	Fc Receptor
FCS	Foetal Calf Serum
GM-CSF	Granulocyt Macrophage-Colony Stimulating Factor
H2	Histamin receptor II
HBSS	Hank's Buffered Salt Solution

HLA	Human Leukocyte Antigen
HPF	High Power field
IL	Interleukin
IFN	Interferon
IV	Intra-Venous
LAK	Lymphokine Activated Killer cell
LC	Langerhans cell
LGL	Large Granular Lymphocyte
LN	Lymph Node
LPS	Lipopolysaccharides
M-CSF	Macrophage Colony Stimulating Factor
MHC	Major Histocompatibility Complex antigen
MLC	Mixed Leukocyte Culture
MTP	Muramyl Tri Peptide
MTX	Methotrexate
NK	Natural Killer cell
PAP	Prostatic Acid Phosphatase
PBL	Peripheral Blood Lymphocyte
PBS	Phosphate Buffered Saline
PMN	Polymorphonuclear
RBC	Red Blood Cell
SCC	Squamous Cell Carcinoma
TA	Tumour Antigen

TCR	T-Cell Receptor
TIL	Tumour Infiltrating Lymphocyte
TL	T-Lymphocyte
WBC	White Blood Cell

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CHAPTER 1

LITERATURE REVIEW

1. 1. Historical Background

A role of the immune system in host resistance against cancer was suggested late last century following the recognition of immunity to bacterial infection. The concept of treating cancer with tumour cell vaccines and sera arose in Germany in 1880 after successful immunization against infectious diseases (Currie 1972). At the turn of this century many attempts at using tumour cells as a vaccine to treat patients with advanced malignant disease were described, the following being typical examples. In 1902 Leyden *et al* used autologous tumour vaccine to immunize patients with advanced cancer, and slight but not significant improvement was claimed for some patients. Coca *et al* (1909) reported "softening and disappearance" of tumour masses in three patients following administration of tumour cell vaccine. In 1909 Dungern *et al* injected patients with their own tumour cells. The appearance of oedema and redness at the injection site were attributed to a specific reaction against tumour cells. Following these encouraging results Coca *et al* (1912) studied a large number of patients with advanced tumours who had been treated with autologous or allogeneic tumour cells. However the authors stated that, according to the results "active

immunization against malignant tumours in human beings is impracticable". Some studies reported negative results following immunization with either autologous or allogeneic vaccine (Risley *et al* 1911; Pinkuss *et al* 1913).

Treatment of cancer by passive immunotherapy was attempted using sera from animals immunized by tumour cells. The results were "promising" with clinical improvement of the patients being reported in some of these studies (Vaughan 1914; Berkeley 1914). Kellock *et al* (1922) was the first to use X-rays to irradiate tumour cells before vaccination. This aimed to reduce the risk of tumour implantation whilst retaining the immunizing value of the cell. However, in this trial no therapeutic effect could be demonstrated. Tissue extract was also used as a vaccine following extensive degradation of tumour cells (Rubens-Duval 1932). Frozen autologous tumour tissues were used to immunize cancer patients by subcutaneous implantation (Stone *et al* 1955). In an attempt to enhance the immunogenicity of tumour cells autologous vaccine mixed with adjuvant or antigenic proteins was used in some trials (Graham *et al* 1959,1962; Czajkowski *et al* 1966, 1967; Cunningham *et al* 1969).

1. 2. Immunosurveillance

The "immunosurveillance" theory was advanced by Burnet in 1970. He proposed that one of the important functions of the immune system in mammals was the detection of non-self antigen, a function he attributed mainly to thymus-

dependent lymphocytes. The role of thymus-dependent lymphocytes in immunosurveillance was based on a study that showed that thymectomized animals were unable to reject transplanted tumours. However providing these animals with lymphocytes from normal donors led to elimination of these tumours (Law 1969). Further evidence appeared to be the observation that mice infected with an oncogenic virus were susceptible to cancer development if these animals were pre-treated with anti-lymphocyte serum (Hirsch *et al* 1968).

Moller (1975) and Schwartz (1975) have discussed the controversy over immunosurveillance. Criticism of this theory arose from the fact that the increased incidence of tumours in thymectomized animals in the previous studies had no relationship to spontaneous development of tumours. In a study of 11,000 nude mice over 4 months none of the mice developed spontaneous tumours (Rygaard *et al* 1974). One year later another study criticized this finding, because many spontaneous tumours developed in nude mice over two years, the average age of tumour development being around 14 months (Outzen *et al* 1975).

Penn (1976) recorded that the incidence of malignant neoplasms in immunocompromised patients was 5-6%, 100 times greater than that in the general population. A high proportion of these tumours were of lymphoid origin. However according to the immunosurveillance concept a wide variety of tumour types should arise. The incidence of the most common tumour in women (breast cancer) does not increase significantly in immunocompromised patients

(Schwartz 1975; Moller *et al* 1975). Blohme *et al* (1984) in their study of renal transplant patients receiving immunosuppression therapy, reported that malignant and pre-malignant skin lesions increased 3-fold in transplanted patients compared to the control population. Several immune mechanisms have been suggested as playing a role in proposed immunosurveillance. Ioachim (1976) demonstrated lymphocyte infiltration at the tumour site and suggested a role for antibodies because of identification of plasma cells in the infiltrate. The role of T cells in immunosurveillance was supported by Antonia *et al* (1996) following the finding that malignant transformed cells express T-cell co-stimulatory molecule B7-1 which activates T cells after encountering a tumour cell. In an animal model it was demonstrated that T-cells mediated immunosurveillance in an MHC-dependent manner (Yamasaki *et al* 1996; Trivedi *et al* 1997).

Baldwin (1977) attributed immunosurveillance to NK cells since NK cells from normal individuals were found to be cytotoxic to tumour cells and more cytotoxic than NK cells taken from individuals with cancer. The ability of these cells to lyse target cells in non-MHC-restricted manner and without previous sensitization strongly suggested this cell as a likely candidate for an immunosurveillance function (Cristoforoni *et al* 1994). Depletion of NK cells in mice led to significantly increased growth of transplanted tumours. This was taken to indicate that these cells were involved in an immunosurveillance mechanism" (Martiniello *et al* 1996). Renneboog *et al* (1996) reported a case of spontaneous disappearance of a neoplastic clone following the withdrawal of

Azathioprine (an immune-suppressive agent), the remission in this case was attributed to the recovery of immunosurveillance. MHC antigen may play an important role in immunosurveillance, down-regulation of this antigen on tumour cells being associated with development and invasion of tumours (Garrido *et al* 1997; Vora *et al* 1997). Recently Dudley *et al* (1996) reported that the incidence of gastrointestinal polyps did not change in severely immunodeficient mice. The absence of immunosurveillance might be due to the lack of immunogenicity in the polyps. These studies suggest a role for cell-mediated immunity in tumour rejection.

1. 3. Evidence of Immunological Reaction to Tumour Cells

Tumour infiltration by lymphocytes, macrophages and plasma cells occurs in both animal and human tumours. Handley (1907a) was among the first to correlate the regression of tumours with inflammatory response in patients with malignant melanoma. Moreover, Handley referred to "body resistance" to tumour cells in the early stages of the disease (Handley 1907 b).

Many studies since then have suggested that infiltration of tumours by leukocytes is associated with better prognoses and tendency to spontaneous regression. Black *et al* (1953,1958) showed that in patients with breast cancer the presence of sinus histiocytosis (distension of the lymph node sinusoid by large cells of mononuclear phagocytic type) in the regional lymph nodes of primary

cancers is associated with a better prognosis. Tumours with marked sinus histiocytosis are associated with a lower incidence of metastases (Anastassiades *et al* 1959). Takraklides *et al* (1974) showed that high T-cell:B-cell ratio is associated with a better prognosis. A well documented case of long-term spontaneous regression of malignant melanoma with evidence of host immune resistance was reported by Bulkley (1975). Histological examination of the lesions revealed lymphocyte infiltration and tumour necrosis. Analysis of TIL isolated from regressive melanoma revealed *in situ* clonal expansion which strongly suggested that antigen driven selection underlay the clonal expansion at the tumour site (Ferradini *et al* 1993; Sensi *et al* 1997). TIL display MHC-restricted cytotoxic activity against autologous tumour cells *in vitro* and most probably contribute to tumour regression (Mackensen *et al* 1994, 1997). Several TSA which are recognised by autologous TIL have been reported and the genes coding for these antigens have been identified (Coulie *et al* 1994; Mori *et al* 1996; Wagner *et al* 1997; Durrant *et al* 1997).

1.4. Tumour Antigen

Tumour specific antigen (TSA) is a constituent of tumour cells that is capable of evoking an immune response specifically to the tumour cells. Most candidate TSA have eventually been found on other non-malignant tissues. For example, certain gangliosides have been detected on melanoma, but have also been shown on the cell membrane of other tissues, especially brain (Pukel 1982).

Antibodies against these antigens are detected in serum from cancer patients and at very low levels in normal individuals (Watanabe *et al* 1982).

Many abnormal proteins are produced by tumour cells that cannot be recognised by the immune system. Normally occurring proteins may be produced in abnormally high amounts by some tumours. TSA can be used as a tumour marker (Chabanas *et al* 1997), the presence of tumour markers may be detected prior to clinical detection of the primary lesion so that screening for these may lead to early tumour detection and treatment with better prognosis. The level of such antigens may be correlated with the prognosis (Goldman *et al* 1993; Yamazaki *et al* 1993). Such markers may be also used as an indication of response to treatment (Carrel *et al* 1973), development of recurrence (Rose *et al* 1993) or as an indication of the activity of the disease (Dreyfuss *et al* 1992; Segal *et al* 1997; Moretti *et al* 1997). Furthermore, down-regulation or up-regulation of certain antigens might correlate with transformation of normal cells to tumour cells (Nollau *et al* 1997).

1. 4. 1. Types of Tumour Antigens

I. Differentiation Antigens

Differentiation antigens are found in normal cells that have a common foetal origin or in cells during a particular phase of their differentiation, {for

example the surface antigens found on T-cells and their precursors during differentiation from stem cell to mature cell (Foon *et al* 1982)}, or on dividing cells but not on resting cells, {for example receptors for interleukin-2 (Uchiyama *et al* 1981)}. Most of the antigens that have been identified on malignant cells are found on normal cells during differentiation or on tissue of the same origin. Some melanoma antigens which are differentiation antigens can be detected on foetal or adult melanocytes (Houghton *et al* 1982). Daar (1981) showed that the presence of differentiation antigen on breast tumour cells reflects the degree of tumour cell differentiation. Some tumour cells express differentiation antigens which are not found in the tissue of origin but on another type of tissue (Daar *et al* 1982).

II. Oncofoetal Antigens

Oncofoetal antigens are normally found in normal foetal tissues but in only small amounts in adult tissues. These antigens can also be found in small amounts in benign tumours or in the sera of normal subjects (Moore *et al* 1971; Chu *et al* 1972; Roushlahti *et al* 1972; Reithdorf *et al* 1997).

Carcinoembryonic antigen (CEA) was first demonstrated in rabbits immunised with human colonic cancer. The rabbits developed antibodies that reacted with foetal-type colonic mucosa but not with normal mucosa (Gold *et al* 1964). Using sensitive assays Chu *et al* (1972) reported that small amounts of this antigen were detected in normal serum. Elevated serum levels of this antigen are

found in patients with other varieties of carcinoma and in patients with non-neoplastic diseases (Moore *et al* 1971).

III. Chemically Induced Tumour Antigens

Abelev *et al* (1963) found that chemically induced hepatoma in mice synthetised protein similar to alpha-globulin found in the sera of foetal and neonate mice but not in adult mice. The characteristic feature of chemically induced tumours is the diversity of antigenicity (Klein *et al* 1960). Diversity of the antigenicity could also be recognised within primary tumours induced by the same carcinogen in the same individual (Globerson *et al* 1964). The incidence and antigenicity of carcinogen-induced tumour relates directly to the concentration of the carcinogen and the frequency of exposure (Prehn *et al* 1975).

Chemical carcinogen may affect the expression of tumour cell protein. Carcinogen reduces some surface proteins, while other proteins show higher expression when compared to normal cells. Some proteins had been detected only in the treated cells but not in normal cells. These new proteins may act as TSA (Altevogt *et al* 1985).

IV. Virus-Induced Tumour Antigens

It has been shown in experimental animals that DNA viruses have

oncogenic potential. Some of these viruses had been suggested as a causative agent of certain human neoplasms, for example the association between EBV and Burkitt's lymphoma (Klein *et al* 1975), and herpes simplex human papilloma viruses and cervical carcinoma (Smith *et al* 1979; Yamada *et al* 1997). In a virally transformed cell, viral gene can be incorporated into the DNA of the transformed cells and then translated by the cell ribosome into protein which can incorporate into the cell membrane of infected cells. This protein could act as a TSA and become a target for T-cells (Svedmyr *et al* 1975; Iezzi *et al* 1997). A significant feature of virus-induced tumours is that tumours induced by the same virus have common antigens. These antigens can induce rejection of the transplanted tumour in syngeneic hosts immunised against the inducing virus (Sjogren *et al* 1961). The similarity of tumour antigen induced by the same virus occurs regardless of the morphology of the tumour and the species of the animal model (Klein 1968, Levitskaya *et al* 1995).

V. Major Histocompatibility Complex Antigens (MHC)

Doherty *et* al (1975) were among the first to refer to MHC and its role in cell mediated immunity. This antigen in humans is represented by human leukocyte antigen (HLA). Class I HLA is found on most nucleated cells (Daar *et al* 1984a). Class II HLA was thought to be present on cells only related to the immune system, however this antigen was also detected in non-lymphoid epithelial tissues (Daar *et al* 1984b). The expression of MHC antigen on tumour

cells varies. Some tumour cells lose MHC antigen expression which is found in the tissue of tumour origin (Carrido *et al* 1976). Other tumours may express new MHC antigen which is found usually on other types of tissues (Wilson *et al* 1979). Some murine tumours express MHC antigen which is normally found in other species (Parmiani 1980). The expression of this antigen on tumour cells was found to play an important role in development of tumour immunity (Fossati *et al* 1984; Guo *et al* 1997; Chang *et al* 1997, Yang *et al* 1997b).

The importance of MHC antigen in tumour immunity comes from the relationship of cytotoxic T-cell activity and the expression of this antigen on tumour cells. The reactivity of cytotoxic cells is restricted by an antigen encoded in the genes of class I MHC antigen (Zinkernagel *et al* 1979; Mackensen *et al* 1997). Moreover, effective resistance against tumour cells by T cell mediated cytolysis cannot be achieved if the tumour cells have lost MHC antigen (Tursz *et al* 1977; Guo *et al* 1997). Virus may modulate the expression of class I antigen on the infected cells. In EBV-carrying Burkitt's lymphoma the expression of such antigen is reduced, which may contribute to the resistance of these cells to cytotoxic T-cells. Expression of MHC antigen in EBV-infected lymphoblastic cell lines make these cells more susceptible to effector cells (Jilg 1991).

The degree of MHC expression might reflect the tumourigenicity and metastatic potential of malignant cells, since the metastatic potential is increased if the expression of MHC is reduced on the surface of the malignant cell (Karre *et*

al 1986; Abdel-Wahab *et al* 1997; Conway *et al* 1997). Down-regulation of MHC in tumour cells correlates with disseminated disease and could be a mechanism by which tumour cells escape immunosurveillance (Van Driel *et al* 1996). The correlation of the magnitude of expression of MHC antigen and the differentiation of the tumour cells has been investigated. Poorly differentiated tumour cells are associated with reduced density of MHC antigen whilst in well differentiated tumour cells the expression of MHC antigen is normal (Tomita *et al* 1990; Esteban *et al* 1990; Conway *et al* 1997).

This observation extends to include the importance of this antigen in recognition of TSA, since a strong relationship was detected between the immunogenicity of tumour cells and the expression of MHC antigen (Baetselier *et al* 1980; Abdel-Wahab *et al* 1997). CTL recognise TSA in association with MHC antigen and the cytotoxic effect of CTL against tumour cells is determined by the presence of specific MHC antigen (Traversari *et al* 1992; Coulie *et al* 1994; Peiper 1997; Yang *et al* 1997b; Chang *et al* 1997). It has been shown that the role of CTL in immunosurveillance is an MHC antigen-dependent mechanism since MHC antigen-positive tumour cells are rejected by CTL while MHC antigen-negative tumour cells can grow progressively in the brain (Yamasaki *et al* 1996; Vora *et al* 1997).

1. 5. Cells Involved in Immune Response against Tumour

Many reports have appeared describing cellular immune responses against cancer. Early studies indicated that this anti-tumour activity is specific (Currie *et al* 1972; Akiyama *et al* 1983). However immune cells can kill tumour cells in a non-specific manner (Mantovani *et al* 1979). Infiltration of tumour tissues by immune cells such as macrophages, plasma cells, DC and lymphocytes indicates a host anti-tumour response (Elston *et al* 1973; Si *et al* 1996; Maehara *et al* 1997). Tumour infiltration by immune cells has been correlated with a favourable prognosis (Matsuda *et al* 1990; Zeid *et al* 1993; Berd *et al* 1997).

1. 5. 1. Lymphocytes in Anti-tumour Immunity

The anti-tumour effect of lymphocytes is attributed mainly to T cells rather than B cells (Pape *et al* 1977). The recognition of cellular antigen by T cells is quite different from the recognition of the antigen by antibodies. T cells recognize cellular antigen in association with MHC antigen on the cell surface (Zinkernagel *et al* 1979; Peiper *et al* 1997; Mackenson *et al* 1997). Blocking of class I HLA by monoclonal antibody prevents the recognition of autologous TSA by T-cells (Anichini *et al* 1985). The recognition of allogeneic tumour cells is not prevented by blocking this MHC antigen (De Vries *et al* 1984; Coulie *et al* 1994; Yamasaki *et al* 1996). However others have shown that MHC antigen is involved in recognition of allogeneic antigen (Yang *et al* 1997b).

The subset of T-cells which has cytotoxic effect is the cytotoxic T-lymphocyte (CTL). CTL were first recognized to cause lysis of target cells in a mixed lymphocyte reaction *in vitro* (Cerottini *et al* 1974). Study of T-lymphocytes (TL) later revealed that these cells can kill tumour cells and virus-infected autologous cells including oncogenic viruses (Zinkernagel *et al* 1975; Gooding *et al* 1983; Mukherji *et al* 1983).

CTL can be isolated from the peripheral blood lymphocytes (PBL) of cancer patients. These cells were found to be cytotoxic to autologous tumour cells *in vitro* after activation and culturing with autologous tumour cells (Anichini *et al* 1985; Mukherji *et al* 1986), allogeneic tumour cells (Zarling *et al* 1976; Vancy *et al* 1981), mixed leukocyte culture (MLC) (Zarling *et al* 1979) and EBV transformed B cells (De Vries *et al* 1984). These studies indicate that peripheral blood lymphocytes (PBL) contain the precursor cells which are cytotoxic to autologous tumour cells.

CTL can also be isolated from tumour infiltrating lymphocytes (TIL) which may be obtained from tumour specimens by mechanical or enzymatic treatment. PBL are more cytotoxic against tumour cells than TIL (Mukherji *et al* 1986). Some studies have reported that these cells are more cytotoxic than PBL when taken from highly antigenic tumour (Brunner *et al* 1981). Following the separation of either PBL or TIL, these cells may be expanded *in vitro* by interleukin-2 (IL-2), while maintaining their cytotoxic function (Mukherji *et al*

1983; Topalian *et al* 1989). Analysing T cell receptors (TCR) of the lymphocytes infiltrating regressive melanoma revealed that there is clonal expansion of TIL which display MHC antigen-restricted cytotoxic activity against autologous tumour cells and which is associated with tumour regression (Mackensen *et al* 1994; Ferradini *et al* 1993; Chang *et al* 1997; Mackenson *et al* 1997).

Some studies have shown that the cytotoxic effect of CTL against tumour cells is specific. TIL with specific anti-tumour activity express CD4 and CD8 markers (De Vries *et al* 1984; Topalian *et al* 1989). CD4 antigens play a role in CTL antigen recognition (Spits *et al* 1982). There are two major sub-populations of T cells: CD8+ (T suppressor/cytotoxic) and CD4+ (helper/inducer: Reinherz *et al* 1981). These TL derived from peripheral blood or infiltrating lymphocytes interact with autologous tumour cells through a T-cell receptor (TCR)-dependent mechanism (Maccalli *et al* 1994; Vondrys *et al* 1997). TL which carry these receptors are responsible for tumour immunity (Mackensen *et al* 1994; Frey *et al* 1996)

The immune response mediated by lymphocytes is a result of interaction between different sub-populations of T-cells. Although the cytotoxic activity of T-cells was thought to be due to of CD8+ CTL, CD4+ CTL are also cytotoxic to autologous tumour cells, i.e. CD4+ and CD8+ T-cells are functionally heterogeneous, e.g. T-cells within CD8 contain cytotoxic as well as suppressor cells and cells within the CD4 sub-population induce suppression or could be

cytotoxic (Mukherji *et al* 1986; Frey *et al* 1996).

While some experiments show that generation of CTL is independent of T-helper cells (Inaba *et al* 1987), others indicated the need for T-helper cells in CTL generation (Ramarli *et al* 1984). Apasove *et al* (1994) referred to the role of CD4+ cells in generation of CTL in MHC I-antigen deficient mice. Treating these mice with anti-CD4 monoclonal antibodies led to abrogation of anti-tumour response. On the other hand, other experiments have shown that tumour rejection is mainly a function of CD8+ but not CD4+ cells (Ramarathinam *et al* 1994; Zhai *et* al 1996; Guo *et al* 1997; Yang *et al* 1997b).

Activation of T-helper cells and generation of CTL require interaction with specific antigen presented in combination with MHC antigen (Topalian *et al* 1996; Mackenson *et al* 1997) as well as other stimulatory signals from antigen presenting cells (Schwartz *et al* 1990; Linsley *et al* 1997; Boussiotis *et al* 1997; Chaux *et al* 1997; Brossart *et al* 1997). Thus if an antigen is presented to CTL without the appropriate co-signals CTL may become anergic. It has been found that this co-signal is delivered to CTL through the B7 surface molecule, present on the surface of dendritic cells (DC), macrophages and activated B and T cells and blocking of this interaction leads to inhibition of immune response (Linsley *et al* 1993; Boussiotis *et* al 1996). Human tumour cells transfected with the B7 gene may simulate CTL in the absence of T helper cells and accessory cells, whilst non-transfected cells fail to produce a response (Dohring *et al* 1994;

Ramarathinam *et al* 1994). Immunization of mice with tumour cells express the B7 molecules led to significant amplification of cytotoxic effect of CD+ TL (Johnston *et al* 1996).

1. 5. 2. Dendritic Cells in Anti-Tumour Immunity

Dendritic cells (DC) were described more than a century ago by Paul Langerhans in a section of human epidermis. Langerhans thought that this cell was related to neural tissues, because it could be stained with gold chloride (Langerhans 1868).

DC in the epidermis are distinguished from other epidermal cells by their clear cytoplasm, the convoluted appearance of the nuclear membrane due to the presence of deep clefts and their dendritic shape. Electron microscopy shows that DC are characterised by the absence of desmosomes and the presence of granules which appear as linear structures with rounded ends ("tennis-racquet"), which are known now as Birbeck granules (Birbeck *et al* 1961). These 'tennis racket'-like granules are specific for this cell. In addition, well developed endoplasmic reticulum and Golgi complex are characteristic, with many lysosomes present in the cytoplasm (Zelickson 1965).

DC are derived from bone marrow (BM: Frelinger *et al* 1979, Katz *et al* 1979), and may share a common pro-generator with macrophages (Volc-Platzer *et*

al 1984; Austyn *et al* 1993). These cells spread from BM to lymphoid and most non-lymphoid tissues, and a migratory form of these cells is found in peripheral blood and afferent lymph (Metllay *et al* 1989).

DC are devoid of surface markers which are specific to NK cells, macrophages, T-cells and B-cells (Austyn *et al* 1987). DC express high quantities of class II MHC antigen which is essential for antigen presentation to T cells. In animal models it was reported that up-regulation of class II MHC antigen on DC occurs following antigen administration. This up-regulation of MHC correlates directly with the ability of DC to present an antigen to T cells (Hopkins *et al* 1989). The density of DC in tumour tissues correlates directly with the expression of class II MHC antigen by tumour cells (Bigotti *et al* 1991).

DC are a heterogeneous group of cells that vary in both phenotype and function. The main function of DC is to present foreign antigen in complex with MHC to T cells, and to deliver an activating signal to T cells. DC in the thymus present self antigen-MHC antigen complex to developing thymocytes to induce tolerance to self-peptide (Austyn *et al* 1987; Metllay *et al* 1989; Linsley *et al* 1997; Boussiotis *et al* 1997; Chaux *et al* 1997; Brossart *et al* 1997).

The role of DC in chemically induced cutaneous tumours has been investigated in many animal studies. Application of 7,12-dimethyl benzo-a-anthracene (DMBA) led to a decrease in the number of DC, the density lowest

when the tumour was well developed. Cessation of carcinogen application was followed by an increase the number of DC to a normal level, which was associated with regression of the tumour (Muller *et al* 1985). These carcinogens may prevent the development of any potential immune response against transformed cells by affecting the number and the function of DC. This reflects the role of DC in anti-tumour immunity. On the other hand, application of benzo-a-pyrene (BP) was accompanied by increased number of epidermal DC (Langerhan's cells, LC). Tumour induced by BP is also characterised by the presence of high number of DC (Ruby *et al* 1989). Tobacco smoke condensate (TSC) application is associated with an increased number of DC. However the morphology and the function DC were abnormal. Following the development of tumours cessation of TSC application was associated with reversible changes of DC morphology and function, increased number of DC, regression of tumours and tumour necrosis (Zeid *et al* 1995). Viruses promote the development of tumour in the same way as chemical carcinogen i.e. diminished numbers of LC leads to decreased local immune-surveillance, and subsequently development of the cancer (Younes *et al* 1968; Caorsi *et al* 1984; McArdle *et al* 1986). Abnormal distribution and function of DC were also described in spontaneous tumours (Gatter *et al* 1984; Egan *et al* 1986; Stene *et al* 1988; Wilson 1991).

The infiltration of lung tumour with dendritic cells was found in patients with adenocarcinoma, squamous cell carcinoma and type II alveolar cell carcinoma. The density of DC in well or moderately differentiated carcinoma was

higher than those with poorly differentiated tumours (Furukawa *et al* 1984; Nakajima *et al* 1985). The association of DC with well differentiated adenocarcinoma and the better prognosis of these tumours may be due to presence of tumour antigen on the differentiated tumour cells attracting DC to the tumour site. Some studies have shown that there is a correlation between the histological stage of the tumour and the density of DC infiltration (Matsuda *et al* 1990; Maehara *et al* 1997).

Clinical studies indicate that the regression or progression of tumours can be correlated to the number of DC infiltrating cancer tissues. In general, growing tumours are accompanied by decreased density of DC (Furukwa *et al* 1985; Nomori *et al* 1986; Tsujitani *et al* 1987; Maehara *et al* 1997). The prognosis in tumour-bearing patients can be correlated with the density of DC in patients with colorectal carcinoma, gastric carcinoma, papillary carcinoma of the thyroid and oesophageal carcinoma (Schroder *et al* 1988; Ambe *et al* 1989; Tsujitani *et al* 1990; Matsuda *et al* 1990).

DC, as an antigen presenting cell (APC), plays an important role in initiating the immune response against specific antigen (Stingle *et al* 1978, Sertl *et al* 1986; Hopkins *et al* 1989). DC participate also in the immunity against neoplasms by presenting TSA to other immune cells (Grabbe *et al* 1991, 1992). TSA presentation by DC is an important step in initiating an adoptive anti-tumour immunity, this immunity is antigen specific and capable of rejecting tumour cells

carrying that antigen (Celluzzi *et al* 1996; Ellem *et al* 1997).

Infiltration of tumour tissues with such cells can serve as an indicator to the ability of tumour cells to evoke an immune response (Ambe *et al* 1989). This ability might be due to the presence of TSA. Recognition of TSA by this cell initiates an immune response against tumour cells carrying this antigen, this immunity is mediated mainly by CTL (Bakker *et al* 1995; Zitvogel *et al* 1996a, c; Van-Schooten *et al* 1997; Mayordomo *et al* 1997). Correlation was found between DC and infiltration of tumour tissues by other immune cells especially lymphocytes (Watanabe *et al* 1983; Drijkoningen *et al* 1987; Ambe *et al* 1989; Zeid *et al* 1993). High numbers of DC were found in tumours infiltrated by other immune cells.

1. 5. 3. Natural Killer Cells in Anti-Tumour Immunity

The cytotoxic effect of natural killer (NK) cells was first recognised following the observation that lymphocytes from the thoracic duct of dogs with transplanted kidneys were cytotoxic to the donor kidney tissues (Coverts 1960). The cytotoxicity of these cells is non-MHC-restricted. NK cells from normal subjects also have a cytotoxic effect on tumour cells. This type of cell mediates cytotoxicity against tumour cells which is non-adoptive and non-MHC restricted, and was named "natural cytotoxicity", and the effector cells mediating this activity were termed natural killer cells (NK). Anti-tumour effects are not the only

function of NK cells. These cells also play a role in immune regulation, immunity against microbial agents such as viruses, and other functions (Trinchieri *et al* 1984; Trinchieri 1989; Robertson *et al* 1990).

Some authors have suggested that NK cells could be any lymphoid cells from non-immunized individuals which mediate non-MHC restricted cytotoxicity (Ritz *et al* 1988). However others have suggested that NK cells represent a new subset of leukocyte which may have its own lineage (Lanier *et al* 1986). NK cell maturation occurs in the absence of functional thymus, and functional NK cells are found in nude mice and severely immune deficient mice (Dorshkind *et al* 1985).

The cytotoxicity of NK cells can be measured in various ways, the most frequent method using K562 target cells. This cell line was derived from a patient with chronic myelogenic leukemia in blastic crisis (Lozzio *et al* 1976). The identification of NK cells depends on the ability of these cells to mediate spontaneous cytotoxicity. However, this function is shared by other types of cells such as macrophages and other T-cells. Thus, other features were considered necessary to identify this population of cells, such as cell surface antigen, phenotype and morphology (Lanier *et al* 1986). NK cells were described as Large Granular Lymphocytes (LGL) (Timonen *et al* 1981), however this feature may not be exclusive since not all NK cells have this feature. Assuming the morphology of NK cells as LGL, 3.6-8% of lymphocytes in peripheral blood

were found to belong to this sub population (Trinchieri 1989; Timonen *et al* 1981).

Fc receptors for the Fc portion of IgG are found on most NK cells. These receptors are characterized by low affinity to IgG unlike those found on other lymphoid cells. The Fc receptors are not important for the function of these cells since blocking or modulation of receptors does not affect cell function (Kay *et al* 1977; 1979a; 1979b; Eremin *et al* 1978). Fc receptors are involved in the regularity mechanism of NK cells (Robinowich *et al* 1996).

NK cells have the ability to form rosettes with sheep erythrocytes (Key *et al* 1980). Since rosette formation is a characteristic feature of T cells, NK cells were thought to belong to this lineage (Key *et al* 1977). Unlike T-cells, NK cells have low affinity for sheep erythrocytes (West *et al* 1977). CD4 antigen is not found on NK cells, however some NK cells carry CD8 antigen on the cell surface, an antigen which is also carried by cytotoxic T-cells (Perussia *et al* 1983). NK cells also react with antibodies used as markers for myeloid lineage (Key *et al* 1980). The precise lineage of NK cells remains unknown. Several studies have suggested that these cells are derived from CD34 bone marrow progenitors (Lotzova *et al* 1993; Miller *et al* 1992, 1994). Res *et al* (1996) referred to the role of the thymus in NK cell differentiation.

The role of NK cells in immune defence against malignancy has been

shown in animal models and humans. In the murine system it was found that there is a direct relationship between the activity of NK cells and the progression of primary tumours and metastasis formation (Trinchieri 1989). Many studies have shown peripheral blood natural cytotoxicity to be reduced in patients with cancer (Tsutsi *et al* 1992; Le-Fever *et al* 1991; Lakhadar *et al* 1989; Cunningham *et al* 1981; Hersey *et al* 1979). Moreover, NK cell activity in patients with cancer reflects the subsequent response to chemotherapy (Garzetti *et al* 1994).

Many investigators have shown that the cytotoxic effect of NK cells is not specific. A wide range of tumour cells are susceptible to NK activity. NK cells from homologous species are more effective than that from heterologous species (Roozemond *et al* 1986). Evidence has demonstrated some selectivity in reactivity against target cells (Mason *et al* 1993). The susceptibility of target cells to the cytotoxic effect of NK cells seems to depend on the structure of the cell membrane, since transfer of liposome containing membrane from sensitive cells to insensitive cells makes the later sensitive to NK cytotoxicity (Roozemond *et al* 1986).

Activation of the cytotoxic mechanism on NK cells requires a structure on the target cells other than the structure for cell binding to NK cell (Trinchieri 1989; Trinchieri *et al* 1981). There is little information about specific receptors used by NK cells for recognition and killing of target cells. CD54 molecules on the surface of NK cells are important for the cytotoxic effect of these cells against

some tumour cell lines (Cristoforoni *et al* 1994).

Primitive cells have been shown to be more sensitive to NK cells than mature cells. For example NK cells lyse undifferentiated but not differentiated embryonic carcinoma cells (Stern *et al* 1980). Some tumour cell lines which are resistant to lysis by polymorphonuclear cells (PMN) and MHC non-restricted T cells are sensitive to NK cells. Several studies have suggested the involvement of MHC in NK cell-mediated cytotoxicity with inverse correlation described between expression of this molecule on the target cell and the susceptibility to cytotoxic effect of NK cells (Pointek *et al* 1985; Jiang *et al* 1996). Increase in the expression of MHC on the target cells by interferon (IFN) leads to decreased sensitivity to the action of NK (Cristoforoni *et al* 1994). NK cells express cell surface receptors for MHC. Interaction of these receptors with self MHC leads to inhibition of cytolytic activity of NK cells (Andrea *et al* 1996; Fry *et al* 1996)

The activity of NK cells can be modulated by several agents, the most well known being IL-2. Culture of NK cells in media containing IL-2 augments the activity of these cells against target cells (Lotzova *et al* 1987). IL-12 was found to have a synergistic effect with IL-2 (Rossi *et al* 1994). Lymphokine activated killer cells (LAK) which mediate non-MHC restricted cytotoxicity were reported to be NK cells (Trinchieri 1989).

NK cells may play a major and important role in host immunity against

cancer and in immunosurveillance against tumour cells (Yamasaki *et al* 1996; Martiniello *et al* 1996), since the action of these cells does not require pre-exposure to target cells. The finding that some tumour-bearing hosts have depressed NK cell activity has led to the suggestion that activated NK cells could be used in immunotherapy of cancer.

1. 5. 4. Macrophages in Anti-Tumour Immunity

Macrophages are a heterogeneous group of cells that participate in many aspects of the immune response. These cells are required for development of specific immunity mediated by other immune cells. In addition macrophages may play a major role in immunity against cancer in general, and against metastatic tumours specifically (Evans *et al* 1972; 1973).

Tumour infiltration by macrophages may reflect the role of this cell in tumour defence, rather than purely ingestion of necrotic materials within tumour tissues. This is supported by the fact that macrophages infiltrate necrotic and healthy tumours to the same degree (Evans 1972). Moreover, tumour infiltration by macrophages correlates with the biological behaviour of tumours, that is, macrophage infiltration inhibits tumour growth and tumours heavily infiltrated by macrophages are less likely to metastasize (Svenning *et al* 1979; Eccles *et al* 1974).

Defective macrophage function has been found in patients with cancer. Treatment of these patients surgically or with chemotherapy enhances the function of macrophages (Kleinerman *et al* 1980; Normann *et al* 1979). The ability of tumour cells to metastasize has been correlated to tumour cell sensitivity to macrophage effects, i.e. the tumour cells most sensitive to macrophages are the least likely to metastasize (Miner *et al* 1983).

Macrophages may affect tumour cells by two different mechanisms: inhibition of tumour cell proliferation (cytostasis) and tumour cells lysis (cytolysis). The cytostatic action of macrophages are not specific to tumour cells, since both normal and transformed cells can be affected by this mechanism (Keller 1976). The effect of macrophages on cell proliferation is mediated by its action on DNA synthesis (Krahenbuhl *et al* 1977; 1980).

The lysis of tumour cells by macrophages is mediated by two mechanisms, antibody-dependent and antibody-independent. The antibody-independent macrophage cytotoxicity was first described by Alexander *et al* (1971). This action of macrophages required contact with the target cells, but the cytotoxicity does not involve phagocytosis of the target cells (Hibbs *et al* 1972; Mantovani *et al* 1977). Following this specific contact, the non-specific reaction takes over, whereby macrophages secrete cytolytic factors which mediate the cytotoxic effect (Johnson *et al* 1981). The binding of macrophages to tumour cells is a specific process mediated by specific receptors on tumour cells (Marino *et al* 1981).

Antibody-dependent macrophage cytotoxicity is lysis by macrophages of target cells which are coated by specific antibodies. The recognition and binding of macrophages to the target cells is mediated through Fc receptors on macrophages, which bind the Fc portion of immunoglobulin. Different types of these receptors have been recognised: FcRI, FcRII and FcRIII (Diamond *et al* 1981; Unkeless *et al* 1981). These receptors have different functions. FcRI and FcRII mediate antibody-dependent cellular cytotoxicity, but FcRIII does not play a direct role in this mechanism (Graziano *et al* 1987).

Peripheral monocytes can be activated by many regulating factors to become macrophages with anti-tumour activity. Granulocyte macrophage-colony-stimulating factor (GM-CSF) and IFN stimulate monocytes *in vitro* to have anti-tumour activity against tumour cells. Unlike GM-CSF, activation by IFN requires another signal such as lipopolysacharides (LPS) to have this action (Grabstein *et al* 1986). Monocytes from normal individuals can be rendered tumourcidal following activation by muramyl tripeptide (MTP), with cytotoxic effect on many tumour cell lines but not to normal cells (Kleinerman *et al* 1983). Moreover monocytes derived from cancer patients activated by MTP and LPS showed anti-tumour cytotoxicity against various tumour cells while sparing normal cells. No cell type was found to be resistant to tumourcidal activity by activated macrophages (Galligioni *et al* 1993). Macrophage colony stimulating factor (MCSF), GM-CSF and IL3 enhance antibody dependent and antibody-independent cellular cytotoxicity of macrophages, the strongest activator being

MCSF (Young *et al* 1990). Macrophages activated by systemic lymphokines can eradicate lymph node and lung metastatic lesions in animal models (Fidler *et al* 1982). However some sub-populations of macrophages may have a negative effect on the immune system (Fu *et al* 1990).

Cytotoxic macrophages may be the treatment of choice for certain malignant lesions, especially those tumours which resist or escape other modalities of treatment. Destruction of tumour cells by macrophages is not selective and resistance of tumour cells within the primary or metastatic lesion does not develop regardless of the heterogenicity or antigenic properties of the tumour cells (Fogler *et al* 1985; Fidler *et al* 1985; Galligioni *et al* 1993).

1. 6. Tumour Immunotherapy

Immunotherapy of cancer is based on the concept that tumour cells have tumour antigens which can provoke an immune response specifically against these cells. In general, immunotherapy has been found to be effective against small tumour masses rather than large tumour masses, thus tumour immunotherapy is usually used together with other types of treatment. Immunotherapy of human cancer was suggested over a century ago before TSA had been identified. Today with identification of TSA by monoclonal antibodies this type of treatment has become the objective of many clinical trials. Different approaches have been attempted to enhance the immune response in tumour-

bearing hosts, either specifically against tumour cells or by generalized activation of the immune system.

1. 6. 1. Active specific immunotherapy

Active specific immunotherapy of cancer is based on the concept that there is tumour specific antigen or antigens (TSA) present on tumour cells during tumour development. These antigens are recognised as non-self and are able to stimulate an immune response. In a cancer-bearing host these antigens may be present, however, they fail to elicit an immune response.

A tumour vaccine consists of intact tumour cells or purified extracts of tumour cells, which may undergo procedures *in vitro* in an attempt to enhance the immunogenicity of the preparation. This preparation can then be inoculated into patients with cancer. Autologous tumour cells are obtained from tumour tissues following surgical resection. Allogeneic tumour cells are obtained from tumours of the same type but from different patients, this form of vaccine depends on the concept that tumour cells of the same histological type have a common TSA. Many attempts have been made to augment the immunogenicity of tumour vaccines, either by modulating these preparations or adding another antigenic materials. These modifications are outlined below.

Many trials of cancer immunotherapy have used an adjuvant to enhance

the immunogenicity of tumour cells (Hoover 1984, 1985). The efficacy of this form of modification of tumour cells has been tested in animal models by immunizing the animal with modified cells and challenging them by the same but unmodified tumour cells. The survival of animals treated with modified cells was longer than those treated with non-modified irradiated cells (Wang *et al* 1967; Apffel *et al* 1966).

The antigenicity of tumour cells may be modified by different methods. Some enzymes may affect the antigen itself in a way that decreases its immunogenicity whilst other enzymes may enhance immunogenicity (Pincus 1981; Seigler *et al* 1979). Alternatively tumour cell antigenicity may be modified by viral infection. Infected tumour cells bear the viral antigen which is highly immunogenic, in addition to TSA. The presence of viral antigen in the tumour cells enhances the presentation of TSA to the immune system (Boone *et al* 1978). High antibody titre against TSA and positive delayed-type hypersensitivity (DTH) were found in mice immunized by recombinant virus contains the coding sequence for TSA (Hu *et al* 1988).

Isolated cell membrane may have the same antigenicity as the whole cell (Neildhart *et al* 1988). Mitchell *et al* (1988) immunized patients suffering from malignant melanoma with mechanical lysates of two melanoma cell lines mixed with adjuvant. Identification of TSA by monoclonal antibodies (Mitchell *et al* 1986) has enabled isolation and synthesis of TSA (Longenecker *et al* 1987;

Blume *et al* 1994)

Trials of Active Specific Immunotherapy

Trials of specific immunotherapy had begun by the turn of this century. The lack of serious complications and cytotoxic effects of this approach to tumour therapy made it possible to carry out trials in humans. The vaccine in early immunotherapy trials consisted of tumour cells, tumour cell suspension or tumour tissues emulsified or macerated and injected into the skin, subcutaneous tissue or abdominal wall (Von Leyden *et al* 1902; Coca *et al* 1909; Risley *et al* 1911; Kellock 1922). Frozen autologous tumour tissues were used in an attempted to prevent tumour implantation while preserving the antigenicity of the cells (Stone *et al* 1955).

Graham *et al* (1959,1962) used an autologous vaccine formed from a supernatant of tumour cells and Freund's adjuvant, in an attempt to enhance the immunogenicity of the tumour cells. Graham reported some improvement in patients with squamous cell carcinoma (SCC). Tumour cell extract mixed with adjuvant was also used to treat patients with different malignancies. Some patients showed a subjective response which could be correlated with DTH reaction of the skin to the tumour cell extract (Hughes *et al* 1964).

Finney *et al* (1960) immunized cancer patients with tumour homogenate

from autologous tumour tissues mixed with Freund's adjuvant. Rise in antibody titres were seen in all patients. Some patients showed an inflammatory reaction in the subcutaneous lesions, and this reaction was followed by regression of the tumours. These changes correlated with the rise of antibody titre.

Depending on the concept that histologically similar tumours have common tumour antigen or antigens, a patient with renal cell carcinoma was vaccinated with tumour cells taken from another patient. Histological examination of the tumour showed no effect of the vaccine, but some precipitate against donor tumour could be detected, which could indicate antibody formation against autologous tumour cells (Nairn *et al* 1963).

In an attempt to enhance the immune response to tumour vaccine, tumour cells were coupled to highly antigenic human gamma globulin. Induction of an immune reaction against this antigenic protein provoked immune response against the coupled cell. Mice with induced squamous cell carcinoma and spontaneous adenocarcinoma were injected with this coupled autologous tumour cell vaccine. Retardation of tumour growth, necrosis, infiltration with lymphocytes and fibrosis of the tumour were seen in the test group, especially those with SCC (Czajkowski *et al* 1966). The same principle was used to create a vaccine for human trials, the results were promising, with regression of the tumour in some patients (Czajkowski *et al* 1967; Cunningham *et al* 1969).

The effect of tumour vaccine on tumour cells may be mediated through formation of cytotoxic lymphocytes or cytotoxic antibodies against these cells. Currie *et al* (1971) found that the cytotoxic effect of lymphocytes to autologous melanoma cells is enhanced by tumour vaccine consisting of irradiated autologous tumour cells. On the other hand some studies showed that autologous immunization enhanced anti-melanoma antibody formation, the extent of the response correlated with the number of tumour cells in the vaccine (Ikonopisov *et al* 1970).

Irradiated autologous tumour cell vaccine was reported to have negative results in patients with malignant melanoma. The survival rate was lower in the immunized group compared to the control (McIllmurry *et al* 1978). McIllmurry stated that this sort of treatment may enhance tumour growth. On the other hand prolongation of the survival of patients with melanoma treated with allogeneic melanoma cell vaccine was reported in another study (Morton *et al* 1978).

Some trials used Bacillus Calmette Guerin (BCG) as adjuvant with tumour cell vaccine to increase the reactivity of the immune system against tumour vaccine (Morton *et al* 1978). Other trials used Corynebacterium parvum (C. *Parvum*) as adjuvant due to complications associated with the use of BCG (McCune *et al* 1979). Complete and partial response of metastatic lesions were reported following treatment with autologous tumour vaccine plus C. *parvum*. (McCune *et al* 1981).

The efficacy of immunotherapy consisting of BCG and irradiated tumour cells treated with neuraminidase was reported by Seigler *et al* (1979). Neuraminidase was used to expose tumour antigen to the immune system. Two cases of renal cell carcinoma with poor prognosis (stage IV) showed complete remission following treatment with autologous tumour antigen mixed with BCG, and another two patients had partial remission (Neidhart *et al* 1980).

Guinea pigs with induced metastatic hepatocarcinoma were immunized with irradiated tumour cell vaccine and BCG. Examination of pulmonary foci showed mononuclear infiltration in the early stages, and histiocyte infiltration with fibrosis and central necrosis was noticed later in the test group (Hanna *et al* 1981).

Morales *et al* (1980) proved the effectiveness in an animal model of immunization by irradiated whole cell or cell extract vaccine. Another study in an animal model showed that immunization with BCG and irradiated tumour cells eradicated dermal tumour deposits and prevented lymph node metastasis (Yarkoni *et al* 1982).

Delayed Type Hypersensitivity reaction (DTH) is used to measure the cellular immunity against specific antigen, including tumour antigen. Immunization of patients with autologous colorectal carcinoma cells and BCG increased DTH significantly toward the tumour cells whilst DTH towards normal

mucosa did not increase (Hoover *et al* 1984; 1985). Complete remission in three patients and partial remission in another six patients with stage IV renal carcinoma was reported following treatment with autologous tumour cells after palliative nephrectomy (Scharfe *et al* 1986).

Mechanical lysates of two melanoma cell lines with a novel adjuvant were used as a vaccine to treat patients with malignant melanoma. Regression of the tumours was noted in eight patients out of twenty-two immunized. Cytotoxic activity of the peripheral blood leukocytes could be detected in these eight patients, unlike unresponsive patients (Mitchell *et al* 1988). In contrast to these promising results, Lamm *et al* (1991) reported that irradiated tumour cell vaccine may enhance the growth of murine bladder cancer and decrease the efficacy of BCG.

The concept of enhancement of antigen presentation by conjugation with a strongly immunogenic hapten has been adopted by some researchers. Twenty-four patients with malignant melanoma were treated with autologous tumour vaccine conjugated with hapten. Fourteen patients had clinical evidence of inflammation of the melanotic lesions, and the rest of the patients showed histological evidence of inflammation (Berd *et al* 1991). Berd stated that the immune response against melanoma antigen was highly augmented by the hapten.

Encouraging results of tumour vaccine were described by Slingluff *et al*

(1992). Slingluff treated 2,968 patients with stage I melanoma with immunotherapy consisting of either three cell lines or a single cell line. The ten-year survival rate was greater in immunized patients compared to the control group. In addition serological activity of immunized patients increased 4.4 fold after immunization. Polyvalent multiple tumour cell vaccine was used in patients with melanoma by Morton *et al* (1992). The five year survival rate increased two to three fold in the treated patients. Most of the patients showed a prompt response to this vaccine. The enhancement of immune response in patients was correlated with clinical response. Berth *et al* (1994) reported increased DTH and cytotoxic T-cell reactivity in patients immunized with polyvalent melanoma vaccine.

The use of genetically modified tumour cells as a vaccine is another approach to enhance the efficacy of tumour vaccine. Tumour cells transfected with cytokine genes were used to increase the ability of the immune system to recognize TSA. The idea behind these trials is to maintain high levels of cytokines at the tumour site, whilst preventing the side effects of high doses of the cytokines. Immunization with transduced tumour cells which express murine GM-CSF resulted in potent long-standing and specific anti-tumour immunity and regression of metastatic lesions (Dranoff *et al* 1993; Wakimoto *et al* 1996; Ellem *et al* 1997). In another trial tumour cells have been transfected with other cytokines such as interleukin-2 and interferon genes (Abdel-Wahab *et al* 1994a, 1994b). It is reported that this method of cytokine production is more effective

than systemic administration of the cytokines (Belldegrun *et al* 1993). Transfection of poorly immunogenic tumour with cytokines increased immunogenicity and decreased tumourigenicity of tumour cells and induced specific immune response against tumour cells (Zitvogel *et al* 1996b; Hollingsworth *et al* 1996).

Autologous melanoma cells conjugated with hapten dinitrophenyl (DNP) induced inflammatory responses in metastatic lesions which was associated with tumour regression. DTH to tumour cells developed in most of the patients (Berd *et al* 1994; Sato *et al* 1995; Berd *et al* 1997; Sensi *et al* 1997). Vaccine consisting of antibodies which mimic TSA administered to patients with ovarian carcinoma resulted in development of specific humoral and cellular immune responses (Wagner *et al* 1997). In an animal model it has been shown that immunization with tumour cells which express co-stimulatory molecules such as B7 induce tumour specific immunity to autologous and HLA-matched allogeneic tumour cells (Yang *et al* 1997b).

1. 6. 2. Active Non -Specific Immunotherapy

Active non-specific immunotherapy aims to increase the immunological reactivity of the host, either humoral or cellular, in a non-specific manner. These therapies may increase immunological activity by creating an inflammatory reaction which enhances the reactivity between effector cells and antigens, or they

may modify the reactivity of the immune cells.

Many agents have been used for this purpose, such as BCG (Stjernsward 1966; Houchens *et al* 1973), C *parvum* (Woodruff *et al* 1966; McCune *et al* 1978), other bacterial preparations such as polynucleotides (Alexander *et al* 1971), and viruses (Herberman *et al* 1977a). Lipopolysaccharides of *Proteus valgaris* are also found to have anti-tumour effect in an animal model (Mizuno *et al* 1968).

Pearl (1929) was among the first to find a relationship between active tuberculosis infection and malignancy. Pearl reported that the incidence of cancer among patients with tuberculosis was less than the normal population.

The immunological properties and anti-tumour effect of BCG have been studied in both animal models and humans. BCG was found to have a number of immunological effects: it increases the number of antibody-producing cells, enhances humoral immunity against unrelated antigen and enhances cellular immunity (Stjernsward 1966; Morton *et al* 1978; Houchens *et al* 1973). In tumour-bearing hosts BCG was found to augment cell mediated immunity (Gutterman *et al* 1973), augment macrophage production (Fisher *et al* 1974) and increase NK activity against tumour cells (Herberman *et al* 1977b). Pre-sensitization of peripheral blood mononuclear cells by BCG had a cytotoxic effect on target tumour cells (Bohle *et al* 1993).

BCG immunotherapy aims to increase the immunological reactivity of the host in a non-specific manner and thus enhance immunity against tumours. The effectiveness of BCG as a treatment depends on the immunogenicity of the treated tumour and the immunological status of the tumour-bearing host (Creau *et al* 1980).

BCG can be administered by different routes: intravenous, intra-muscular, intra-lymphatic and intratumoural. Intravenous administration of BCG can inhibit the development of pulmonary metastasis in animals injected with rat sarcoma (Baldwin *et al* 1973). The inhibitory effect of BCG on tumour development was also demonstrated in animals with primary tumours (Baldwin *et al* 1974).

To avoid the side effect of systemic administration of BCG, BCG was given by scarification to patients with stage III and IV melanoma. Reduction of relapse rate and improvement of survival were reported (Gutterman *et al* 1973). Morton *et al* (1974) reported regression of 90% of melanotic nodules injected with BCG, whilst 17% of uninjected nodules underwent regression. Intra-pleural BCG improved the survival of patients with stage I disease (McKneally *et al* 1976; 1977).

In animal models it was shown that IV administration of *C. parvum* could delay the appearance of tumours, whether the bacteria was injected after or before inoculation of tumour cells (Woodruff *et al* 1966). In mice, intra-dermal

administration of *C. parvum* led to transient inhibition of chemically induced tumour (Currie *et al* 1970). Likhite (1974) referred to the effectiveness of subcutaneous administration of *C. parvum* in animal models. Prolongation of the survival of mice with fibrosarcoma injected intravenously with *C. parvum* was also reported (Milas *et al* 1974). Pimm (1977a) demonstrated the effectiveness of local rather than intravenous administration of *C. parvum.*

Intra-lymphatic administration of BCG was applied to patients with gynaecological malignancies. Patient survival correlated directly with the inflammatory reaction in lymph nodes due to BCG injection (Mangan *et al* 1977). Promising results were reported with intravenous and intra-vesical administration of BCG following incomplete resection of the tumour in patients with transitional cell carcinoma (morales *et al* 1981; Herry *et al* 1983). BCG treatment also enhanced the effect of subsequent chemotherapy in animals (Hanna 1982). BCG has been used to inoculate the site of the vaccination prior to vaccine administration. This approach was attempted to enhance the immune reaction to the tumour cells at the site of the injection (Livingstone *et al* 1983; 1985).

Apart from treatment of superficial bladder cancer by intra- vesicular BCG alone, most of the human trials have used BCG as an adjuvant to tumour cell vaccine or antigen preparation to treat patients with colorectal cancer (Hoover *et al* 1984; 1985), metastatic melanoma (Berd *et al* 1988; 1990; 1991), leukemia (Powles *et al* 1973; Rittch *et al* 1978; Rosenberg *et al* 1978) and experimental

bladder cancer (Morales *et al* 1980; Lamm *et al* 1991, 1995; Bui *et al* 1997).

Patients with renal cell carcinoma developed DTH to tumour cells following vaccination with autologous tumour cells mixed with BCG (Galligioni *et al* 1997). In clinical trials BCG administered intravesically was effective in producing immunity against transitional cell carcinoma of bladder and increased the survival in these patients (Lamm *et al* 1995; Morecki *et al* 1995; Bui *et al* 1997). In murine bladder tumour BCG inhibited tumour growth and was associated with signs of tumour regression (Kadhim *et al* 1997).

Other types of bacteria or bacterial products have also been used to augment the immune reaction against tumours. Heat-inactivated Clostridium buyricum produced anti-tumour activity in mice by increasing interferon production and enhancing antibody production (Wang *et al* 1996). Complex polysaccarides isolated from bacteria is able to slow tumour growth in mice (Donmez *et al* 1997).

1. 6. 3. Adoptive immunotherapy

Adoptive immunotherapy refers to the treatment of cancer by administration of lymphoid cells. This type of immunotherapy is a result of the fact that cellular mechanisms play a major role in anti-tumour immune response. The lymphoid cells may be obtained from the same individual (autologous), from

other individuals with tumour or from normal individuals (allogeneic: Powles *et al* 1973). These cells may be sensitized (specific) or not sensitized (non-specific). The definition of adoptive immunotherapy has commonly been extended recently to include treatment with cell products such as interleukins and interferons.

Early trials of adoptive immunotherapy used spleen cells as a source of immune cells. Injection of these immune competent cells into patients with malignancies affected tumour cells by graft-versus-tumour reaction (Woodruff *et al* 1963). Suppression of tumour growth and regression of established tumours were reported in rats treated with intravenous administration of sensitized lymphocytes (Delorme *et al* 1964).

Adoptive immunotherapy can be achieved by bone marrow transplantation. A case of leukemia in progressive phase was treated by bone marrow transplantation from six donors following total body irradiation. One week after transplantation the patient had complete remission for twelve months, chromosomal study showed that leukocytes belonged to the donors (Mathe *et al* 1965). Because large amounts of immune cells are required to eradicate tumour cells, bone marrow from patients with chronic myeloid leukemia were grafted to patients with acute leukemia resistant to chemotherapy (Schwarzenberg *et al* 1966). Some patients achieved remission which was attributed by the author to the anti-leukemia affect of the grafted leukocytes. White blood cells (WBC) from

animals were used in human trials to induce graft-versus tumour reaction (Symes *et al* 1968).

Complete regression of well established Meth A fibrosarcoma in mice could be achieved by IV administration of T-cells from immunized donors (Berendt *et al* 1980). Regression of this highly antigenic tumour could be achieved by adoptive immunotherapy only following depletion of suppressor cells in the recipients. Mills *et al* (1983) showed that sensitized T lymphocytes led to regression of tumour in T cell-deficient recipients but not in normal recipients. This regression was associated with the production of cytolytic T-cells in the recipients. Eradication of tumours is carried out by donor T lymphocytes rather than host cells (Greenberg *et al* 1984).

To identify the sub-populations of T-cells responsible for this therapeutic effect, thymectomized mice with disseminated leukemia were treated with non-cytolytic $CD4^+$ 8^- T-cells which have been sensitized to TSA. These cells which are not directly cytolytic to tumour cells were able to eradicate disseminated leukemia (Geenberg *et al* 1985). On the other hand, purified $CD8^+$ cells which are specifically cytolytic to tumour cells were found to have limited therapeutic effect in tumour rejection. Administration of interleukin-2 (IL-2) with cytolytic T-cells markedly enhanced the effect of these cells (Greenberg *et al* 1986). The function of IL-2 in enhancement of the cytotoxic effect of the effector cells may be through prolongation of the survival of these cells *in vivo*, rather than

generation of new effector cells (Bookman *et al* 1987).

One limitation of adoptive immunotherapy is the requirement of large numbers of sensitized lymphocytes to achieve tumour regression. Lymphocytes can be expanded *in vitro* by culture in supernatant containing IL-2. These cells are more effective in prolonging the median survival time in tumour-bearing hosts compared to uncultured cells. Moreover the specificity of the cultured cells was maintained during the culture. These cells have the ability to cure mice of established disseminated tumour (Cheever *et al* 1981; Eberlein *et al* 1982; Chen *et al* 1990).

TIL can be activated and expanded *in vitro*. by IL-2. Patients with malignant melanoma were treated with these expanded cells by Aebersold *et al* (1991). T-Lymphocytes (obtained from peripheral blood or bone marrow) were also expanded by IL-2 and used for adoptive immunotherapy in animal models and human trials (Murphy *et al* 1993; Porter *et al* 1994). Adoptive immunotherapy may have superiority in treatment of cancer which is resistant to cytotoxic effects of anti-neoplastic drugs (Mooney *et al* 1993). Vaccines consisting of tumour cells genetically modified to produce cytokines are able to elicit specific anti-tumour immunity, decrease tumourogenicity and induce CTL cytotoxicity (Abdel-wahab *et al* 1994a, 1994b; Zitvogel *et al* 1996a; Cayeux *et al* 1997; Vondrys *et al* 1997; Mackensen *et al* 1997).

The critical role played by DC in initiation of adoptive immunotherapy has attracted attention to use DC as a vaccine in tumour-bearing hosts. Reduction of metastatic nodules was reported following treatment with sensitized DC in humans and animal models (Hsu *et al* 1996; Nair *et al* 1997; Yang *et al* 1997b). Vaccines consisting of modified DC to produce certain cytokines or to express surface proteins were also evaluated as adjuvant immunotherapy in animal models (Zitvogel *et al* 1996c; Kim *et al* 1997).

1. 6. 4. Passive Immunotherapy

Passive immunotherapy is the oldest form of cancer immunotherapy. This form of immunotherapy involves administration of a serological component which has a direct or indirect anti-tumour activity. This includes not only antibodies but also complement, lymphokines or other factors in the circulation which have an anti-tumour effect. Most patients with localized malignancy have antibodies in their serum against tumour antigens. Progression of the disease is associated with disappearance of the antibodies from the sera of these patients (Lewis *et al* 1969).

There are three sources of human sera or plasma used in human immunotherapy trials :

1. Plasma from normal donors (Davis 1947; Moore *et al* 1957);

2. Plasma obtained from donors who are immunized with normal or

tumour tissues (Brittingham 1960; Laszlo *et al* 1968).;

3. Plasma taken from patients in remission or who underwent spontaneous regression (Ngu *et a l* 1967; Horn *et al* 1971).

The anti-tumour activity of antibodies can be categorized as:

1. Antibody-mediated cytotoxicity. The effector cells recognize the Fc portion of antibody on the tumour cells which leads to lysis of the tumour cells (Kipps *et al* 1985);

2. Complement-mediated cytotoxicity. The complement cascade is activated by immunoglobulin (Ig) attached to tumour cells. This leads to tumour cell destruction (Burnstein *et al* 1980);

3. Direct effect of Ig on special structures on the surface of tumour cells which plays a vital role in cell survival (Masui *et al* 1984; Change *et al* 1993);

4. Production of anti-idiotypic antibody which have configurations similar to tumour antigen. These antibodies may induce auto-antibodies or cellular toxicity against that antigen (Forstrom *et al* 1983; Saleh *et al* 1993; Wagner *et al* 1997);

5. Antibodies may act as a mediator to deliver other agents to tumour cells (Deguchi *et al* 1986).

Anti-tumour antibodies could be produced in rabbits or sheep sensitized to tumour cells. Injection of experimental animal with tumour cells, together with these antibodies could prevent tumour growth (Buinauskas *et al* 1959).

Regression of malignant melanoma was reported in a patient following whole blood transfusion taken from patients who underwent spontaneous regression (Sumner *et. al* 1960). Ngu *et al* (1967) treated two patients with Burkitt's lymphoma with plasma taken from patients who underwent spontaneous regression. One of the patients had marked anti-tumour effect and the other had moderate improvement.

Hyper-immune gamma globulin may be obtained by immunizing animals with purified antigens from solid cancer or leukemic tissues. Patients with solid tumours and leukemias were treated with this immunoglobulin. Most of the patients with leukemia achieved remission, and all patients with solid tumours had subjective improvement (De Carvalho 1963; Murray 1965; Newman *et al* 1977).

The development of monoclonal antibodies has allowed the use of such antibodies as passive immunotherapy, especially following the identification of tumour antigens. These monoclonal antibodies can be directed against tumour cells without affecting normal cells (Badger *et al* 1983). Mice with transplanted leukemia were immunized with monoclonal antibodies directed against T-cell differentiation antigen (Thy-1.1). Immunization with monoclonal antibodies and complement led to cure of the leukemia in a significant number of the mice (Burnstein *et al* 1980). Patients with cutaneous T-cell lymphoma were treated with murine monoclonal anti-T cell antibodies. Regression of skin and lymph node lesions was reported. Some patients may have developed anti-monoclonal

antibodies against murine antibodies which may be the cause of unresponsiveness in some instances (Miller *et al* 1981, 1983).

While some have shown that murine monoclonal antibodies directed against human tumour antigen can be administered safely to patients with malignancies (Sears *et al* 1982), other studies have reported some side effects of such treatment, for example pulmonary toxicity (Foon *et al* 1984). Formation of blocking factors in the serum of treated patients has also been described (Catane *et al* 1988).

To avoid the hazard of anti-mouse antibody formation, some have tried to develop human monoclonal antibodies (Hale *et al* 1988). Hale reported some improvement in two patients with non-Hodgkin's lymphoma treated with such antibodies. Koda *et al* (1990) generated monoclonal antibodies against colonic cancer using human nodal lymphocytes. These antibodies were found to be cytotoxic to human colonic cancer cells in the presence of complement (Change *et al* 1993).

Antibodies which are directed to the surface antigen of tumour cells can be used as a carrier for cytotoxic or radioactive agents (Vial *et al* 1957; Ghose *et al* 1972, 1975; Deguchi *et al* 1986; Waldmann *et al* 1991). Fragments of monoclonal antibodies were also used in immunochemotherapy. Immunotoxin consisting of Fab fragment of monoclonal anti-CD22 antibodies coupled to

deglycosylated ricin were used to treat patients with B-cell lymphoma (Vitetta *et al* 1991). Partial response was achieved in 38% of the patients. Coupling of antibodies to Staphylococcus *aureus* entertoxin was attempted to direct T-cell activity against tumour cells carrying these coupled antibodies. Administration of this conjugation to mice previously inoculated with lymphoma cells improved the survival in the treated group (Ochi *et al* 1993).

Antibodies which mimic TSA administered to patients with advanced tumour may result in development of cellular and humoral immunity to TSA (Wagner *et al* 1997; Durrant *et al* 1997). Monoclonal antibodies which can bind to tumour cells and to specific receptors on T-cells are able to increase the immunogenicity of tumour cells and generate efficient immune responses (Guo *et al* 1997).

1. 7. Summary

This review shows that the immune system plays an important role in controlling certain malignant growths. Immunological protection against cancer is mediated predominantly by cellular immunity. Tumour infiltration by immune cells and clonal expansion of TIL *in situ* indicates the presence of an immune response against tumour cells. However in tumour-bearing subjects induction and maintenance of a sufficiently large immune response is required for effective immunity against tumour cells. Tumour immunotherapy aims to enhance the

immune response against tumour cells by increasing the immunogenicity of tumour cells, improving presentation of TSA to the immune system or increasing the reactivity of the immune system. This thesis aims to examine a role of DC in detecting vaccine-induced immune responsiveness to induced skin carcinoma in mice, specifically whether DC may be a marker of such a response.

CHAPTER 2

MATERIALS AND METHODS

2. 1. Animals

Male and female BALB/c mice were obtained from the University of Tasmania animal house with approval of the Animal Ethics Committee. The animals were kept in the Department of Pathology animal house and fed food and water *ad libitum.* Mice were between 6-8 weeks old at the beginning of the experiment and were kept in groups of 7-10. The mice were observed closely on a daily basis.

2. 2. Carcinogen Treatment

Benzo-a-pyrene (BP) was obtained from Serva USA (Lot No 30091). Every second week 10 ml of 0.5% BP in acetone solution was prepared (50 μg of BP dissolved in 10 ml acetone). All precautions were taken when working with BP either in preparation or experimentation. Double gloves, surgical mask and laboratory coat were always worn.

Animals were treated in a room specifically for carcinogen use and all waste (sawdust, pipette tips, tissues, etc.) were disposed of in labelled rubbish bags and

burned. The dorsal trunk of each mouse was shaved and 20 µl of 0.5% BP solution applied. Mice were treated twice a week for twenty-four consecutive weeks.

2. 3. Separation of Mice into Test and Control Groups

Following carcinogen application visible changes in the skin were recorded every week. By the end of the twenty-fourth week mice developed either single **(Figure 1, page 89)** or multiple **(Figure 2, page 90)** tumours. After the development of tumours, mice were divided into two groups: control and test. Seven mice were placed in each cage, the cages were given numbers from 1-8. The mice were marked by painting the ears with different colours, for example right ear red cage 1(RR 1), left ear red cage 1 (LR 1), both ear red cage 1 (BR 1). The mice in both groups were chosen to have similar tumours regarding size.

2. 4. Anaesthesia

Mice were anaesthetized by intra-peritoneal injection of 10% phenobarbitone solution (Boehringer Ingleheim). The dose was adjusted according to the weight of the mouse. Mice were kept in a special cage until they were anaesthetized.

Ten percent phenobarbital solution was prepared by adding 5 ml phenobarbital to 45 ml phosphate buffered saline (PBS; Oxoid England Lot R007531-001). The solution was sterilized by passage through a Millipore filter. Five ml aliquots were stored at 4^{o}C.

2. 5. Tumour Excision

Mice were placed on cork boards covered with sterilized tissues. The dorsal trunk and skin surrounding the tumour was shaved with electrical clippers and sterilized with 70% alcohol. A whole tumour in the case of mice with multiple tumours, or part of the tumour in the case of mice with single tumours was excised using scalpel and forceps. Bleeding was stopped by applying a cotton ball at the site of the wound. The wound was then closed with stainless steel auto-clips (**Figure 3, page 91**). Three to seven clips were used for each mouse depending on the size of the wound. All procedures were carried out under sterile conditions.

Tumour was placed in a sterile yellow top universal container containing Modified Hank's Medium (MHM: Sigma, USA, Lot H6136) and transferred to a laminar flow cabinet. The weight of the tumour tissues was recorded.

2. 6. Disaggregation of Tumour

2. 6. 1. Mechanical Disaggregation

In the laminar flow cabinet tumours were transferred to 100 mm petri dishes containing Modified Hank's Medium. The tumours were trimmed of necrotic tissue, fat and other non-tumour tissues and rinsed three times in Modified Hank's Medium. Tumour was then transferred to another petri dish containing an equal

volume of 0.1% trypsin solution (ICN, USA, Lot 47999) and 0.2% EDTA (Univar Australia). Mechanical disaggregation was carried out using scalpel and forceps in a cross-cutting motion until the tumour was fragmented into small pieces (1-2 mm). Care was taken not to exert excessive pressure with the scalpel and forceps and not to scratch the petri dish.

Mechanically disaggregated tumour cells were obtained by collecting the 0.1 % trypsin solution and 2% EDTA solution used to rinse the tumour tissues during mechanical disaggregation. This solution was passed through a syringe containing sterile gauze to remove any lumps. The filtered material was placed into a yellow top sterile universal container and centrifuged at 160 g for 10 minutes. The pellet was resuspended in 0.5 ml of RPMI medium (ICN, USA) after washing the pellet twice with RPMI medium. A sample was taken and cell viability was determined using eosin-Y dye exclusion.

2. 6. 2. Enzymatic Disaggregation

The minced tumour tissues (from 2. 6. 1.) were transferred to a sterile yellow top universal 50 ml container by pipette. Equal volumes of 0.1% trypsin and 0.2% EDTA were added to tumour tissues. Volumes were dependent upon the weight of the tissues, i.e. 10 ml trypsin and EDTA solution per gram of tissue. The tumour tissue was then incubated at 37^{o} C on an orbital shaker (100 rpm) for 40 minutes. Under sterile conditions the supernatant was harvested and fresh trypsin and EDTA

solution added. The tissue was incubated for a further 40 minutes and the above steps repeated. Following harvesting the supernatant was filtered through sterile gauze to remove tissue lumps. Five ml of RPMI was added to the filtered harvest to dilute the enzyme solution. The cell suspension was then centrifuged at 160 g for 10 minutes. The supernatant was discarded and the pellet resuspended in 5 ml RPMI. This step was repeated, and the pellet was resuspended in 500µl RPMI.

2. 7. Sterility

One harvest from each tumour was placed in a culture flask with culture Medium (Modified Hank's Medium). The culture flask was incubated for five days at 37 ^{o} C in a humidified atmosphere of 5% CO_2 in air. Following incubation the flask was checked for bacterial growth.

2. 8. Cell Number and Viability

Cell viability was assessed using eosin-Y dye exclusion (0.1 % in PBS). A 10µl sample of cell suspension was mixed with 10µl of eosin-Y dye, and placed on a haemocytometer. Cells were examined under light microscopy. Viable cells excluded the stain, whilst dead cells did not. The cell count per ml and the viability were calculated according to the formula:

Cells /ml = $N * 2 * (25/X) * 10^4$

where N = Number of the cells counted

X = Number of the squares counted

% viability = No of viable cells / total cells * 100

2. 9. Cryopreservation

All cryopreservation procedures were performed in a laminar flow cabinet using sterile technique. Freezing medium was prepared immediately prior to cell freezing and placed in ice. The freezing medium contained 20% dimethylsulphoxide (DMSO; Sigma USA Lot 1344666), 20% foetal calf serum (FCS: ICN. USA) and 60% RPMI (ICN USA). Cells were suspended and aliqouted in 500 µl into cryopreservation tubes (Simport, Canada) and placed in ice. An equal volume of freezing medium was added dropwise, doubling the volume each time (that is, 1 drop, 2 drops, 4 drops, 8 drops etc.). Tubes were wrapped in cotton wool and placed in a -80°C freezer. Storage time was between one and six weeks.

2. 10. Cell Thawing

Cells were thawed in a 37°C water bath until a small lump of ice remained. Cells were then transferred to a 10 ml centrifuge tube on ice in the laminar flow cabinet. Ten ml RPMI was added gradually. The cells were centrifuged at 160 g for 10 minutes. The supernatant was discarded and the pellet washed twice with Hank's

Buffered Salt Solution (HBSS; Sigma USA). The cell pellet was resuspended in 500 µl of HBSS and cell number and viability determined. An additional 500µl of HBSS was added and the cell suspension taken immediately for radiation treatment.

2. 11. Cell Irradiation

Cells where irradiated at the Holman Clinic, Royal Hobart Hospital. The tubes were kept at room temperature and placed in an open box filled with water to the level of the cell suspension. A cone (70 X 70 mm^2) was placed over the tubes in the box touching the bottom of the box, the test tube directed over the cone. A dose of 25.00 Gy (1 Gy = 100 Rads) was given to the cells over 4.25 minutes. The tubes were then transferred to a laminar flow cabinet to check cell viability.

2. 12. Vaccination

Each mouse was photographed and given a number according to the colour of ear painting and the number of the cage. The weight of each mouse either in control or in the test groups was recorded.

The injection site was shaved with electrical clippers and swabbed with 70% alcohol, and the tumour cells were injected subcutaneously. The site of vaccination was chosen to be away from the tumour to detect any implantation of the tumour cells from the vaccine. The site of vaccination was recorded. The vaccination was

repeated every week for three consecutive weeks. The control group was injected with equivalent volumes of HBSS alone.

2. 13. Tumour Size

Comparison of tumour size between the mice was indicated by measuring the largest diameter of the tumour before and after giving the three vaccines. The diameter was measured by a measuring tape placed beside the tumour whilst photographing the mice.

2. 14. Killing the Animals

By the end of the third week the mice were killed by cervical dislocation. Photographs were taken of each mouse either in the control or test groups showing the tumour clearly. The tumour tissues from the control and test groups were resected and placed in labelled tubes containing formalin, for later histological examination.

2. 15. Histological Examination

Tumour specimens from treated and control mice were surgically excised, fixed in 10% formalin and embedded in wax. The tissue blocks were sectioned at a thickness of 5 μm. Several tissue sections from each tumour (5- 20 sections) were

stained with haematoxylin and eosin. The slides were examined under light microscopy by two persons working together using a 2-head microscope. The degree of tumour infiltration leukocytes and the degree of tumour necrosis were recorded by the observers as mild, moderate and marked. The degree of infiltration and necrosis was categorized as follows :

LEUKOCYTE INFILTRATION:	
mild	< 10 cells / HPF
moderate	10- 50 cells / HPF
marked	> 50 cells / HPF

NECROSIS:	
mild	Necrosis involving < 10 %
moderate	Necrosis involving 10-40%
marked	Necrosis involving > 40%

2. 16. Immunostaining Procedure

Formalin fixed paraffin embedded tissues were placed in freezer for 90 minutes (< 40 ^{o}C). Paraffin sections were cut at 3 microns, picked up on APTS

coated slides **(page 66)** and dried at 56°C for 2 hours and then overnight at 37°C. The pre-stored slides were placed in a 56°C oven for 10 minutes prior to dewaxing. The slides were dewaxed in 2 changes of xylene for 4 minutes each and rehydrated through a descending series of alcohol of 100%, 95% and 70%, 2 minutes each and in running tap water for another 2 minutes and then rinsing in DDW for 2 minutes.

Slides were transferred into plastic slide racks (the racks were completely filled with slides, empty spaces were filled with blank slides), sections were immersed into plastic troughs containing the Target Retrieval Solution (Dako Code No. S 1700). All slides were covered with the solution and troughs were covered with the lids and transferred into microwave at 500 W for 8 minutes. After 8 minutes the racks were agitated few times and microwaved for another 8 minutes at 500 W, then the troughs were removed from the microwave and allowed to cool for 20 minutes at room temperature. Slides were rinsed under running water and transferred into TRIS buffer (0.05M) where they were incubated overnight.

The slides were incubated at room temperature for 60 minutes in primary antibodies (anticow S 100, Dako, Z 311) diluted 1 : 100 in diluent (5% bovine albumin, solution in 0.05 M TRIS buffer, CSL S 31202) , while for negative control slides antiCow S 100 was substituted with negative control reagent (Biogenex Ca 94568) and used at the same dilution. After incubation with primary antibodies slides were washed with 0.05 M TRIS buffer three times five minutes each using a squeeze bottle. The slides were then incubated at room temperature for 60 minutes

with secondary antibodies (biotinylated anti-rabbit immunoglobulin, Biogenex HK 326-UR) at a dilution of 1 : 100 in 5% albumin solution (bovine albumin, CSL S 31202) in 0.05 M TRIS buffer **(page 67).** After washing with 0.05 M TRIS buffer as for primary antibodies slides were incubated for 60 minutes in alkaline phosphatase conjugated streptavidine (Biogenex HK 321-UK) at a dilution of 1 : 40 in 0.05M TRIS buffer.

The slides were washed with 0.05M TRIS buffer for 15 minutes and filtered substrate **(page 68)** was applied for 12 minutes before washing the slides with deionised water to remove the substrate. The slides were then transferred into appropriate staining racks and washed in running water for 2 minutes and then counterstained in Mayer's Haematoxylin for 2.5 minutes. After washing in running water the slides were blued in ammoniated water for 30 seconds and then washed again in running water and dried in a 76^{o}C oven for 10 minutes. The slides were then mounted, overslipped using Eukitt (Carl Zeiss, 00 20 20) and dried on a hot plate for a few minutes.

2. 17. Identification and Counting of Dendritic cells

The dendritic cells were recognised by their S100-positive red cytoplasm, and by cell morphology. Cells were only counted if the nucleus was identified. Other S100-positive material was excluded by their morphology. The degree of dendritic cell infiltration was assessed by two persons separately, each counting five high

power fields (magnification x200) randomly selected from the tumour tissue and adjacent surroundings. An average of ten high power fields was calculated for each section.

2. 18. Sterile Technique

All disaggregation and other procedures were carried out in a biological safety cabinet. Ultraviolet light was switched on for fifteen minutes before using the cabinet. The cabinet was swabbed with 70% alcohol before starting and after finishing each procedure. All sterilized solutions and containers were opened in the cabinet and were closed before taking them out. Sterile forceps were used to take pipettes and the other equipment out of their sterile containers. All pipettes were placed on special racks during the procedure to keep them sterile.

Glass pipettes were plugged with cotton wool and put in aluminium containers which were sealed by autoclave tape. The pipettes were sterilized in an autoclave at 120^{o}C for 20 minutes. The gauze used for filtering the disaggregated cells was prepared and sterilized as follows. The gauze was cut into small pieces (2 cm X 2 cm). The pieces were then folded many times and placed in aluminium foil and sealed by autoclave tape. The pieces were placed into autoclave bags and sterilized in an autoclave at 120^{o}C for 20 minutes. Pipette tips were placed into small glass containers and sterilized in the autoclave as for the pipettes. Surgical instruments were placed in sealed autoclave bags and autoclaved at 120^{o}C for 20 minutes. All

glassware used was washed in detergent followed by rinsing with tap water, then soaked in distilled and double distilled water.

2. 19. Media and Solutions

Hank's Balanced Salt (HBS, Sigma, USA, Lot H6136)

Component	g/L
Calcium Chloride	0.185
Glucose	1.000
Magnesium Sulphate	0.09767
Potassium Phosphate Monobasic	0.060
Potassium Chloride	0.400
Sodium Chloride	8.000
Sodium Phosphate Dibasic	0.04788
Phenol Red Na	0.011

Modified Hank's Medium (MHM)

800 ml of autoclaved water was placed in a 2L flask with a magnetic stirrer. HBSS powder was added to the water and stirred until dissolved. 0.35 g sodium bicarbonate ($NaHCO_3$) was added and the pH adjusted to 7.40 ± 0.05 with HCl (1M) and/or NaOH (1M). The solution was filtered by Millipore filtering (0.45 μm), and

stored in 500 ml lots at 4°C.

Phosphate Buffer Saline (PBS, Oxoid England)

100 ml of autoclaved water was placed in 200 ml flask. One tablet of PBS salt was added to the flask and stirred until it dissolved.

Trypsin Solution 0.1%

To 100 ml of PBS 0.1 g sucrose and 0.1 g trypsin were added. The pH of the solution was adjusted to 7.4 ± 0.05 with HCl (1M) and/or NaOH (1M). The solution was sterilized by millipore filtering (0.2 μm) and stored in 50 ml yellow top universal containers at -20° C.

EDTA Solution 0.2%

EDTA solution was prepared by adding 0.2g EDTA to 100 distilled water. The flasks were placed on an electrical stirrer until EDTA dissolved and the pH of the solution was adjusted to 7. 4 ± 0.05 with HCl (1M) and/or NaOH (1M). The solution was sterilized by millipore filtering (0.2 μm) and stored in 50 ml yellow top universal containers at - 20° C.

RPMI 1640 Medium

RPMI 1640 was obtained from ICN, USA and stored at 4° C. 2.5 ml of Penicillin (5,000 IU/ml)/Streptomycin (5mg/ml, ICN, USA) and 5 ml of 3% L-glutamine were added to 500 ml of RPMI.

Foetal Calf Serum (FCS, ICN, USA)

Foetal calf serum was inactivated by incubation at 56° C for 30 minutes and stored at 4° C.

Freezing Media

10 ml freezing media was prepared each time as follows. Six ml (60%) RPMI 1640 with antibiotic (5 ml/l, Penicillin 5,000 IU/ml and Streptomycin 5 ml/l) was placed in each of several yellow top universal containers and these were placed in an Esky filled with ice. Two ml (20%) FCS was added to the each containers and kept in the Esky until the temperature reduced. Two ml (20%) of DMSO was added to each container whilst on ice and shaken several times.

3-Aminopropyltriethoxysilane (APTS) coating of microscope slides

8 ml of APTS was added to 400 ml of acetone, a microscope slide rack was

loaded with clean dry microscope slides, the slides were immersed in APTS solution for 2 minutes then rinsed with two changes of fresh distilled water. Slides were drained and dried over night at room temperature.

Stock 0.5 M Tris buffer pH 7.6

121.12 gm of Trishydroxy-methyl-amino-methane was dissolved in approximately 1600 ml of deionised water, 180 gm of sodium chloride was added and dissolved and pH was adjusted to 7.6 using 70 ml of concentrated HCl. The volume was made up to 2 L using deionised water.

0.05 M Tris buffer pH 7.6

The working solution of 0.05 M Tris buffer pH 7.6 was made by adding deionised water to 100 ml of 0.5 M Tris buffer pH 7.6 making the final volume up to 1L.

Primary antibodies, antiCow S100 (1 : 100)

2 ml of 0.05 M TRIS buffer

100 µl of bovine albumin (CSL S 31202)

20 µl of antiCow S 100 (Dako, Z 311)

Negative control reagent

2 ml of 0.05 M TRIS buffer

100 µl of bovine albumin (CSL S 31202)

20 µl of negative control reagent (Biogenex Ca 94568)

Substrate

Solution 1:

- 0.2 M Propanediol buffer

    -1.514 gm of 2-Amino-2-Methyl-1,3-propanediol

    (Merck, 801464)

    -72 ml of deionised water

-0.05M Tris-HCL saline buffer pH 9.7

    -1.211 gm of Tris Base (Boehringer No 604203)

    -200 ml of deionised water

-2.4 gm Sodium chloride (Ajax Chemicals-Univar-465)

-0.11 gm of Levamisol (Sigma L 9756)

The propandiol buffer was mixed with the Tris-HCL buffer, sodium chloride and Levamisol were added and the solution was stored in the fridge.

Solution 2:

-10 mg of Naphthol AS-B1 phosphate (Sigma, 2250)

-120 µl of N,N-Dimethylformamide (Sigma, D-4254)

-Naphthol AS-B1 phosphate was dissolved in N,N

-Dimethylformamide in the fume cupboard.

Solution 3:

-40 µl of 5% New Fuchsin

-5 gm of New Fuchsin (Sigma N 0638)

-100 ml of 2N of HCl

-100 µl of 4% Sodium Nitrite

-4 gm of Sodium Nitrite (Ajax Chem-Univar 492)

-100 µl of deionised water

New Fuchsin was mixed with the freshly prepared sodium nitrite in a cold environment (-80°C freezer) for one minute. 19.5 ml of solution 1 was mixed with 120 µl of solution 2, solution 3 was then added and pH was adjusted to 8.7 using 2N HCl. The working solution (substrate) was mixed and filtered.

2. 20. Statistical and Graphical Analysis

The Excel and Macintosh Statview program were used for statistical analysis and graph drawing. The significance (P value) was calculated using the same program> P values of <0.05 was recognised as indicating a statistically significant difference.

CHAPTER 3

RESULTS

3. 1. Treatment with Benzo-a-pyrene

Benzo-a-pyrene was applied in 20 µl volumes twice weekly to the dorsal surface. Two days after carcinogen application visible changes on the skin surface could be seen. These consisted of erythema and hair loss. Four weeks after the application of carcinogen, papillomas had begun to appear. By the tenth week approximately 50% of the mice had one or more discrete papillomas. Fungating tumours started to appear after ten weeks, and by week twenty four almost all mice possessed multiple or single tumours.

The growth of the tumours was not limited to the site of carcinogen application, but occurred at other sites (e.g. neck, abdomen and leg).

3. 2. Surgical Resection of the Tumours

3. 2. 1. Mortality and morbidity

Some mice haemorrhaged during resection of the tumour, especially those

mice with infiltrating tumours. Four mice died one to two hours following the operation as a result of bleeding. Another three were killed during the operation due to uncontrolled bleeding. Those that recovered from the operation proceeded to do well. Most mice returned to normal movement and activity within a day.

3. 2. 2. Wound Healing and Infection

The wound infection rate following the surgical procedure was very low. Two out of fifty mice suffered from infection. In one the infection was severe, and the mouse was killed. Wound healing at the site of the operation occurred within four to seven days. Some mice showed delayed wound healing, especially those with necrotic tumours or with a wide wound surface.

3. 2. 3. Anaesthesia

Phenobarbital at a concentration of 10% V/V was used to anaesthetize the mice. Mice were given one to one and a half times the recommended dose. The mice slept ten to fifteen minutes following the anaesthezia and revived after thirty to fifty minutes. Out of 75 mice anaesthetized for tumour resection only two died as a result of anaesthezia.

3. 3. Disaggregation of Tumour

3. 3.1. Mechanical Disaggregation

Mechanical disaggregation was initially used to prepare the vaccine due to the uncomplicated and fast method. However, mechanical disaggregation of tumour tissues using scalpel and forceps yielded a low percentage of viable cells (30-60 %). This low viability may have been in part due to the aggressive mechanical procedure killing the cells. However tumour tissue is also mixed with necrotic tissue which may have been a contributor to the low cell viability. Thus the viability of mechanically disaggregated cells depended on the condition of the tumour before resection.

Cells obtained by this method were mixed with red blood cells (RBC) and white blood cells (WBC) contributing up to 30% of the total cells. Due to the low viability of tumour cells obtained by mechanical disaggregation and the presence of a high percentage of RBC and WBC, the tumour cells obtained using this method were not used to prepare the vaccine.

3. 3. 2. Enzymatic Disaggregation

Enzymatic disaggregation of tissues yielded a higher cell viability than the mechanical method **(Figure 11, page 103).** The percentage of cell viability obtained

by enzymatic disaggregation ranged from 60% to 90%. The harvest cryopreserved for later use as a vaccine had a viability greater than 80%. Harvests with low viability (< 60%) and large amounts of cell debris were excluded.

The centrifugal force used in harvesting cells was found to be critical for cell viability. Viability decreased and harvests contained large amounts of cell debris when 300g, 240g and 200g were used. The optimum force used was 160 g. The cell viability from the first harvest was always low despite the low centrifugal force (160 g), due to the presence of necrotic tissue. However with successive harvests cell viability increased.

Cells were washed twice with culture media following harvesting to remove the enzymes. Washing the cells did not affect cell viability as the viability before and after washing was not significantly different (**Figure 12, page 104**).

The sterility of the technique was tested by culturing one or more of the harvests for several days to detect any evidence of bacterial growth. The culture flasks were tested every day for seven days. One flask contained bacterial growth which was excluded from the experiment. No bacterial growth could be detected in the other flasks.

3. 4. Cryopreservation

The cells were cryopreserved immediately after preparation to ensure viability was as high as possible. Cryopreservation caused a decrease in cell viability of 10 - 30 %.

3. 5. Cell Irradiation

Radiation of the cells did not affect the viability of the cells significantly (**Figure 13, page 105**). No tumour growth was noticed at the site of vaccine injection which indicated that the X-ray dose was enough to eliminate the ability of the cells to divide and to prevent any growth of implanted cells.

3. 6. Vaccination

Both the control and test groups were vaccinated. The test group was vaccinated with tumour cells mixed with HBSS while HBSS alone was used to vaccinate the control group. Vaccination of the control group by HBSS eliminated the possibility of an HBSS effect on tumour growth and allowed the exposure of both groups to the procedure of vaccination.

3. 7. Site of Vaccination

The vaccine was injected subcutaneously, and the site of the vaccine was chosen to be away from the site of tumour growth. This allowed the detection of any tumour implantation at the injection site. No tumour growth could be detected at the site of vaccination in all mice treated by the vaccine.

3. 8. Infection at Injection Site

No infection could be detected at the site of vaccination in the controls (vaccinated with HBSS) or test group (vaccinated with irradiated cell vaccine in HBSS).

3. 9. Tumour Infiltration by Dendritic Cells

Mice in the tested group were treated with irradiated tumour cell vaccine in HBSS once a week for three consecutive weeks while the control was treated with HBSS alone in identical schedule. The effect of the vaccine on the tumour was measured by comparing the degree of tumour infiltration by DC. The DC average per 10 HPF (magnification x200) ranged from 1.8 to 24.4 cells with the mean of 10.5 ± 6.5 cells in the test group while in control group the DC average ranged from 0.3 to 8.7 cells with the mean of 3.9 ± 2.9 cells, the difference in DC infiltration between the two groups significant at $P < 0.05$ (anova: single factor). The degree of

tumour infiltration by DC in the test group and control group is shown in **Table 7&8 (page 87, 88)**. Dendritic cells were observed amongst the tumour cells and in the stroma surrounding the tumour tissues. DC appeared more rounded with loss of the dendrites **(Figure 7&8, page 99, 100)**.

3. 9. 1. DC Infiltration and Tumour Size

The difference in tumour size before and after administration of the vaccine was calculated by measuring the largest diameter of the tumour **(Figure 3, page 91).** The difference in diameter of the tumour before and after vaccination for the test and control groups is illustrated in **Table 2&3 (page 82, 83).**

No correlation was found between tumour size and the degree of infiltration by DC (correlation co-efficient 0.037 and $P > 0.05$; F-test). Some mice with marked infiltration showed an increase in their tumour size while others had a stable tumour size or a slight decrease in tumour size. The correlation co-efficient between DC infiltration and the difference in tumour size before and after vaccination is 0.123 ($P > 0.05$; F-test). There was no significant difference between the control and the test groups regarding size of tumour. The mean tumour size before and after vaccination in the test group was 13.2 ± 3.9 and 16.2 ± 6.2 respectively ($P > 0.05$; t-test), and in the control group 15.6 ± 5.0 before and 18.0 ± 6.1 after vaccination ($P > 0.05$; t-test).

3. 9. 2. DC Infiltration and Vaccine Dose

Mice received three doses of tumour cell vaccine with an average range between 1.5 and 3.7 X 10^5 tumour cells in the three vaccines (**Table 4, page 84**). No correlation was found between the dose of the vaccine and the degree of tumour infiltration by DC. The correlation co-efficient of dose of tumour cells vs. the degree of tumour infiltration by immune cells was 0.33 and $P > 0.05$ (F-test) indicating no significant correlation between dose of vaccine and degree of DC infiltration of tumours.

3. 10. Tumour Infiltration by non-DC Leukocytes

Other tumour-infiltrating immune cells consisted of lymphocytes, macrophages and neutrophils, however lymphocytes comprised the majority of infiltrating leukocytes. The difference in leukocyte infiltration between the two groups was significant. In 70.5% of the control group the infiltration was mild compared to 23.5% in the test group. On the other hand 53% of the mice in the test group had marked infiltration compared to 12% in the control. The percentage of mice with moderate infiltration in test and control groups was 23.5 and 17.5 respectively (**Table 1, page 81; Figure 4 a,b,c & 9 page 92,93,94 &101**). The difference between both groups was significant ($X^2 = 6.41$ and $P < 0.05$; chi sq.).

Mice in test and control groups were divided into three groups according to the

degree of non-DC leukocytes infiltration: mild, moderate and marked. The mean of DC infiltration in each of the three groups was 4.5 ± 3.8, 7.4 ± 5.5 and 11.1 ± 6.9 respectively, the difference of DC infiltration between three groups was significant ($P < 0.05$; anova: single factor).

Degree of non-DC leukocyte infiltration	**DC infiltration**
Mild (n = 16)	4.5 ± 3.8
Moderate (n = 7)	7.4 ± 5.5
Marked (n = 11)	11.1 ± 6.9

3. 11. Tumour Necrosis

The difference in the degree of necrosis between the control and the test groups was significant. Marked necrosis was detected in 65% of the treated mice compared to 17.5% of controls. Necrosis in the control group was mild in 53.% of mice compared to 17.5% of the treated group. The percentage of mice which had moderate necrosis in test and control groups was 17.5% and 29.5% respectively **(Table 1, page 81; Figure 5 a,b,c & 10 page 95,96,97 & 102)**.The difference between both groups was significant ($X^2 = 6.07$ and $P < 0.05$; chi sq.).

Mice in test and control groups were divided into three groups according to the degree of necrosis: mild, moderate and marked, the mean of DC infiltration in the

three groups was 4.1 ± 3.8, 7.5 ± 7.8 and 9.8 ± 5.4 respectively, the difference of DC infiltration between three groups was significant ($P < 0.05$; anova: single factor).

Degree of tumour necrosis	DC infiltration
Mild (n = 12)	4.1 ± 3.8
Moderate (n = 8)	7.5 ± 7.8
Marked (n = 14)	9.8 ± 5.4

After necrosis of the tumour tissues, sloughing of the necrotic tissue was followed by re-epithelization. This phenomenon was noticed in some of the mice in the test group but not in the control group (**Figure 6, page 98).**

3. 12. Mouse Weight

The mice in both groups were weighed before giving the vaccine and after three weeks from first vaccination to assess the difference in the weight between both groups during this period of time (**Table 5, 6, page 85, 86**). The difference was not significant between both groups ($P > 0.05$) indicating that the vaccine did not have an effect on mouse weight within this period. The mean weight before and after vaccination in the test group was 21.8 ± 2.6 g and 21.5 ± 3.3 g respectively ($P >$

0.05; t-test), and in the control group 22.7 ± 4.3 g before and 22.3 ± 3.9 g after vaccination ($P > 0.05$; t-test).

Degree	Infiltration		Necrosis	
	Test	Control	Test	Control
Mild	4 (23.5%)	12 (70.5%)	3 (17.5%)	9 (53%)
Moderate	4 (23.5%)	3 (17.5%)	3 (17.5%)	5 (29.5%)
Marked	9 (53%)	2 (12%)	11 (65%)	3 (17.5%)
Total	17	17	17	17

Table 1: Number of mice with mild, moderate and marked degree of non-DC leukocytes infiltration and tumour necrosis in test (n = 17) and control (n = 17) groups.

Mouse Number	Tumour Diameter before Vaccination (mm)	Tumour Diameter after Vaccination (mm)	Difference in Tumour Diameter (mm)	% Difference in Tumour Diameter
1	16	10	-6	-37
2	11	16	+5	45
3	21	23	+2	9
4	12	13	+1	8
5	12	14	+2	16
6	11	18	+7	63
7	18	17	-1	-5
8	12	18	+6	50
9	16	14	-2	-12
10	15	17	+2	13
11	10	20	+10	100
12	21	35	+14	66
13	12	10	-2	-16
14	10	7	-3	-30
15	11	14	+3	27
16	10	16	+4	40
17	7	14	+7	100
Mean ± sd	13.2 ± 3.9	16.2 ± 6.2	2.9 ± 5.0	25.97 ± 41.2

Table 2: Difference in tumour size three weeks following vaccination of the test group.

Mouse Number	Tumour Diameter before Vaccination (mm)	Tumour Diameter after Vaccination (mm)	Difference in Tumour Diameter (mm)	% Difference in Tumour Diameter
1	22	16	-6	-37
2	17	17	0	0
3	15	19	+4	26
4	12	22	+10	83
5	18	19	+1	4
6	19	17	-2	-10
7	21	31	+10	47
8	21	24	+3	14
9	20	13	-7	-35
10	14	21	+7	50
11	16	16	0	0
12	12	16	+4	33
13	20	29	+9	45
14	9	9	0	0
15	7	9	+2	28
16	16	18	+2	12
17	6	11	+5	83
Mean ±sd	15.6 ± 5.0	18.0 ± 6.1	2.5 ± 4.9	20.2 ± 34.6

Table 3: Difference in tumour size three weeks following vaccination of the control group.

Mouse Number	First Dose x 10^5	Second Dose x 10^5	Third Dose x 10^5	Average Doses
1	2.7	2.9	1.9	3.0
2	3.8	2.5	2.7	2.53
3	1.5	1.7	1.4	1.53
4	2.0	2.7	3.7	2.63
5	1.9	2.2	3.2	2.43
6	2.1	2.7	1.5	2.10
7	2.5	1.8	2.5	2.26
8	2.9	2.5	3.1	2.83
9	2.3	2.6	2.3	2.40
10	1.4	1.9	1.3	2.53
11	2.1	1.8	3.5	2.46
12	2.4	1.9	2.7	2.33
13	4.1	3.9	2.9	2.65
14	2.3	2.1	3.7	2.70
15	1.6	2.2	1.7	1.83
16	2.9	2.6	2.9	2.80
17	3.7	3.2	3.8	3.56
Mean ± sd	2.5 ± 0.8	2.4 ± 0.6	2.6 ± 0.8	2.5 ± 0.5

Table 4: Number of viable cells in the three vaccine doses.

Tumour cells were irradiated and the viability of the cells was assessed using eosin-y dye exclusion. The number of viable cells was calculated according to the formula:

$$N \times 2 \times (25/Y) \times 10$$

where **N** = Number of the viable cells counted , **Y** = Number of squares counted

Mouse Number	Weight before Vaccination (g)	Weight after Vaccination (g)	Difference in Weight (g)	% Difference in Mouse Weight
1	29	30	1	3
2	20	18	-2	-10
3	24	26	2	8
4	20	19	-1	-5
5	19	20	1	5
6	25	26	1	4
7	22	20	-2	-9
8	20	21	1	5
9	21	22	1	5
10	24	23	-1	-4
11	24	22	-2	-8
12	22	21	1	5
13	21	22	1	5
14	20	18	-2	-10
15	22	21	-1	-5
16	19	17	-2	-10
17	19	19	0	0
Mean ± sd	21.8 ± 2.6	21.5 ± 3.3	-0.2 ± 1.4	-1.2 ± 6.6

Table 5: Difference in mouse weight three weeks following vaccination of the test group.

Mouse Number	Weight before Vaccination (g)	Weight after Vaccination (g)	Difference in Weight (g)	% Difference in Mouse Weight
1	30	28	-2	-7
2	28	27	-1	-4
3	34	32	-2	-6
4	26	26	0	0
5	22	23	1	5
6	22	20	-2	-9
7	20	21	1	5
8	19	17	-2	-10
9	20	21	1	5
10	21	21	0	0
11	23	21	-2	-9
12	19	19	0	0
13	21	22	1	5
14	18	18	0	0
15	24	22	-2	-8
16	19	20	1	5
17	21	22	1	5
Mean ± sd	22.7 ± 4.3	22.3 ± 3.9	-0.4 ± 1.3	-1.3 ± 5.8

Table 6: Difference in mouse weight three weeks following vaccination of the control group.

Mouse No	Field 1	Field 2	Field 3	Field 4	Field 5	Field 6	Field 7	Field 8	Field 9	Field 10	Mean
1	4	7	23	6	6	3	1	4	2	1	5.7
2	0	0	0	0	0	3	2	0	0	1	0.6
3	0	1	6	1	1	6	14	16	0	1	4.6
4	3	2	7	8	9	6	2	6	8	7	5.8
5	7	4	7	4	5	1	3	9	6	13	5.9
6	6	12	3	8	0	13	4	0	0	3	4.9
7	2	2	8	2	2	3	3	0	5	12	3.9
8	0	0	0	1	0	1	0	0	0	1	0.3
9	4	5	11	3	2	15	17	9	12	5	8.3
10	1	0	0	0	0	4	7	3	3	2	2
11	7	8	7	10	9	17	8	6	11	2	8.5
12	4	11	1	5	6	11	28	12	5	4	8.7
13	0	1	0	0	0	2	0	3	5	1	1.2
14	1	1	2	3	1	1	0	0	1	2	1.2
15	0	1	1	0	1	4	0	3	1	0	1.1
16	1	4	2	1	3	2	1	0	2	0	1.6
17	2	1	1	5	6	4	4	0	2	3	2.8

Table 7: Number of DC and the mean in 10 HPF (magnification x200) of each mouse in the control group. The mean ± sd of DC for the control group was 3.9 ± 2.9.

Mouse No	Field 1	Field 2	Field 3	Field 4	Field 5	Field 6	Field 7	Field 8	Field 9	Field 10	Mean
1	11	0	13	32	12	24	14	15	8	2	13.1
2	0	5	3	2	1	16	11	3	9	6	5.6
3	1	9	17	16	21	9	16	10	12	7	11.8
4	0	3	2	6	0	0	1	2	1	3	1.8
5	18	19	4	22	9	24	15	28	16	31	18.6
6	2	1	5	0	1	0	7	5	8	2	3.1
7	5	14	8	19	20	7	16	4	1	28	12.2
8	1	2	5	7	4	0	15	3	0	8	4.5
9	5	12	1	5	17	14	11	7	4	12	8.8
10	25	24	29	14	26	24	11	26	7	9	19.5
11	14	23	16	13	10	14	25	12	6	11	14.4
12	11	9	19	17	2	13	4	20	17	5	11.7
13	4	12	16	18	20	18	0	15	14	9	12.6
14	0	0	1	2	2	9	0	2	5	0	2.1
15	22	34	12	37	31	19	17	19	21	32	24.4
16	26	13	2	6	7	22	8	7	1	4	9.6
17	20	11	0	3	1	4	3	0	5	3	5

Table 8: Number of DC and the mean in 10 HPF (magnification x200) of each mouse in the test group. The mean ± sd of DC for the test group was 10.5 ± 6.5.

Figure 1

Mice treated with benzo-a-pyrene twice a week for twenty-four consecutive weeks which developed a single skin tumours.

Figure 2

Mice treated with benzo-a-pyrene twice a week for twenty-four consecutive weeks which developed multiple skin tumours.

(a)

Before

Vaccination

(b)

After

Vaccination

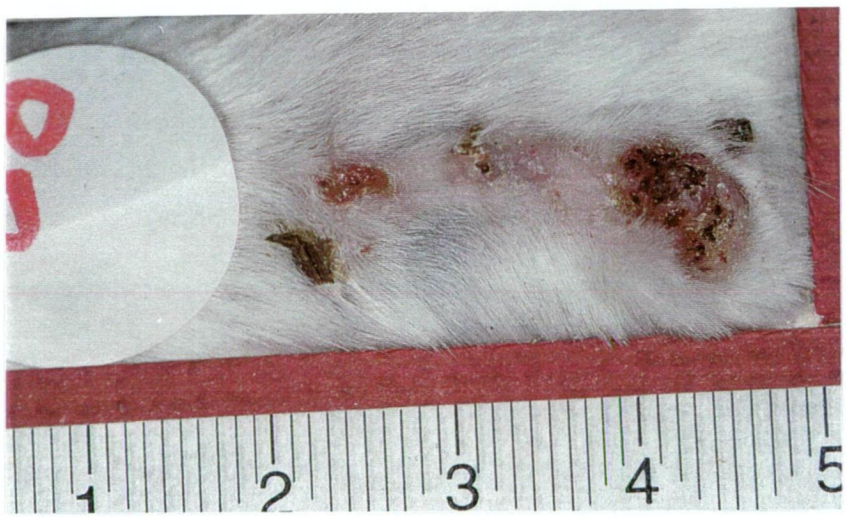

Figure 3 (a & b): Difference in tumour size three weeks following vaccination.

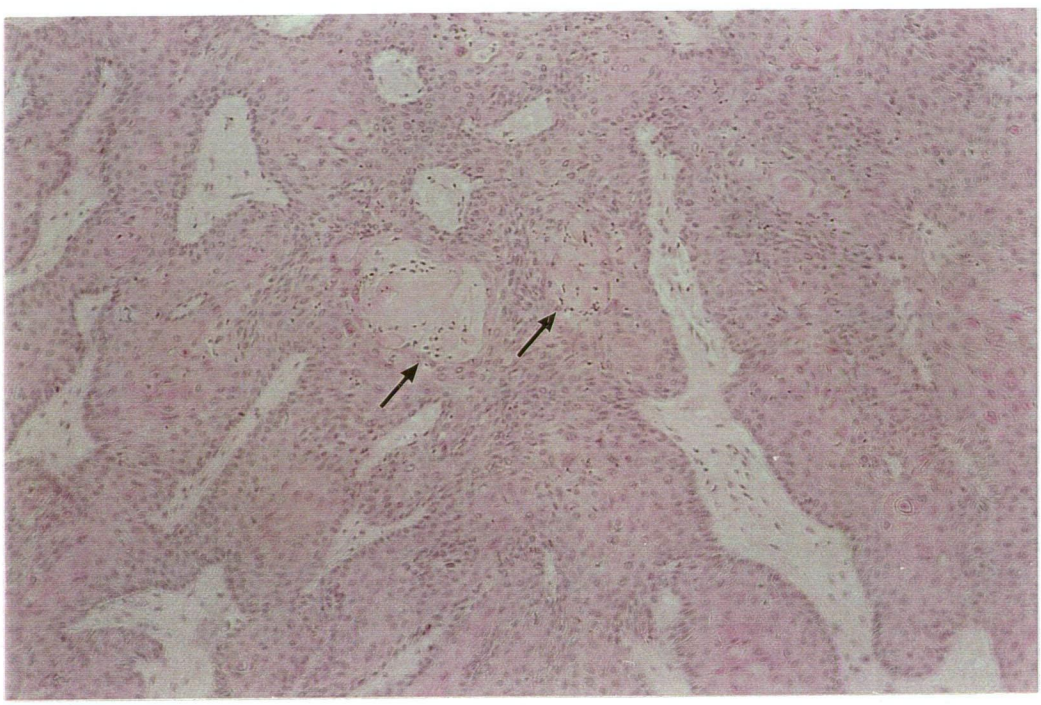

Figure 4 a: Degree of tumour infiltration by leukocytes (mild).

→ = Tumour infiltrating leukocytes

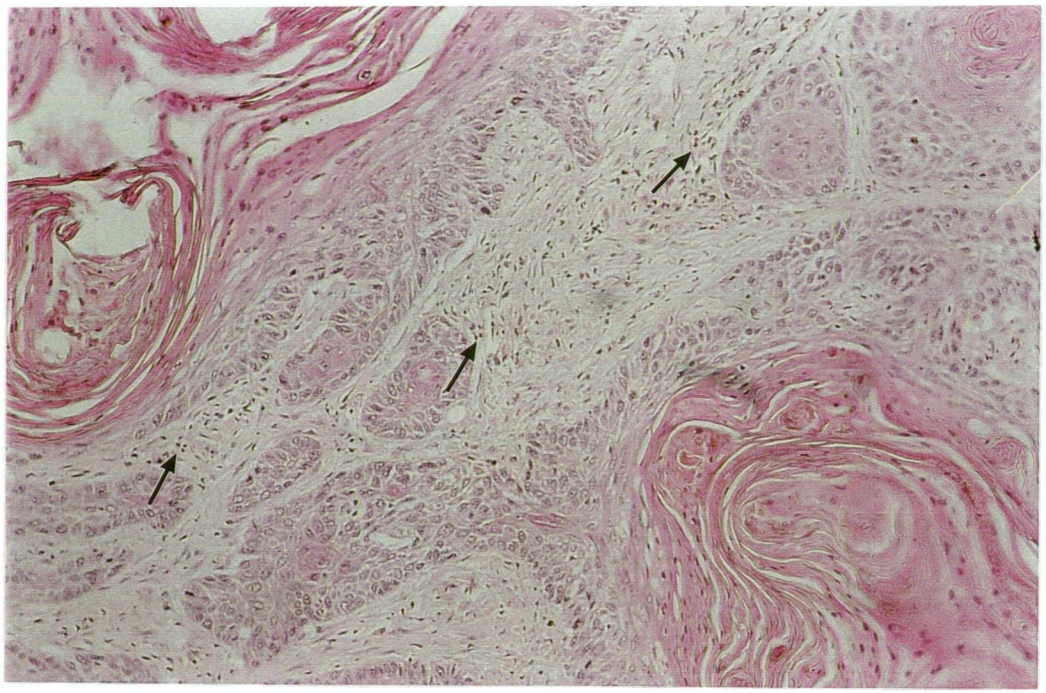

Figure 4 b: Degree of tumour infiltration by leukocytes (moderate).

→ = Tumour infiltrating leukocytes

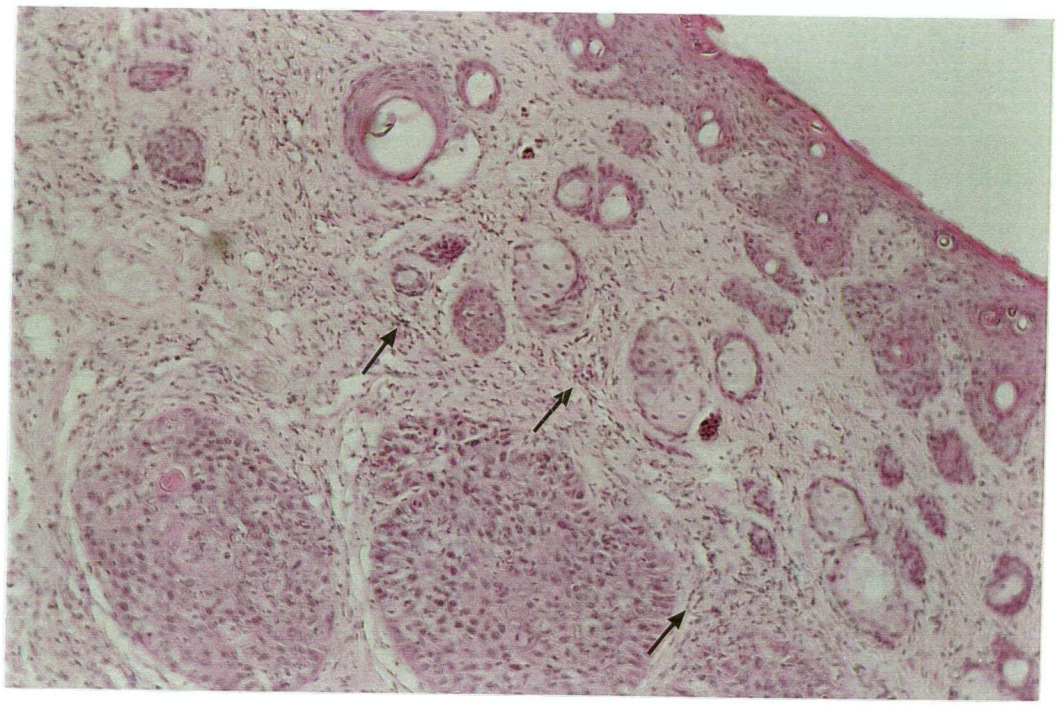

Figure 4 c: Degree of tumour infiltration by leukocytes (marked).

→ = Tumour infiltrating leukocytes

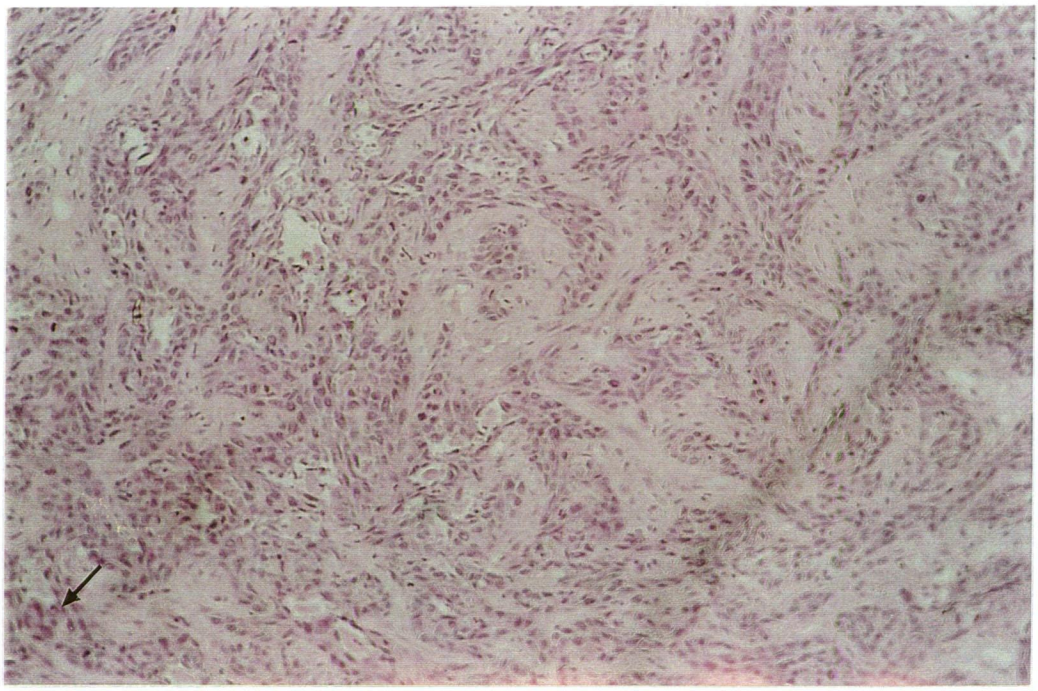

Figure 5 a: Degree of tumour necrosis (mild).

→ = Necrotic tumour cells

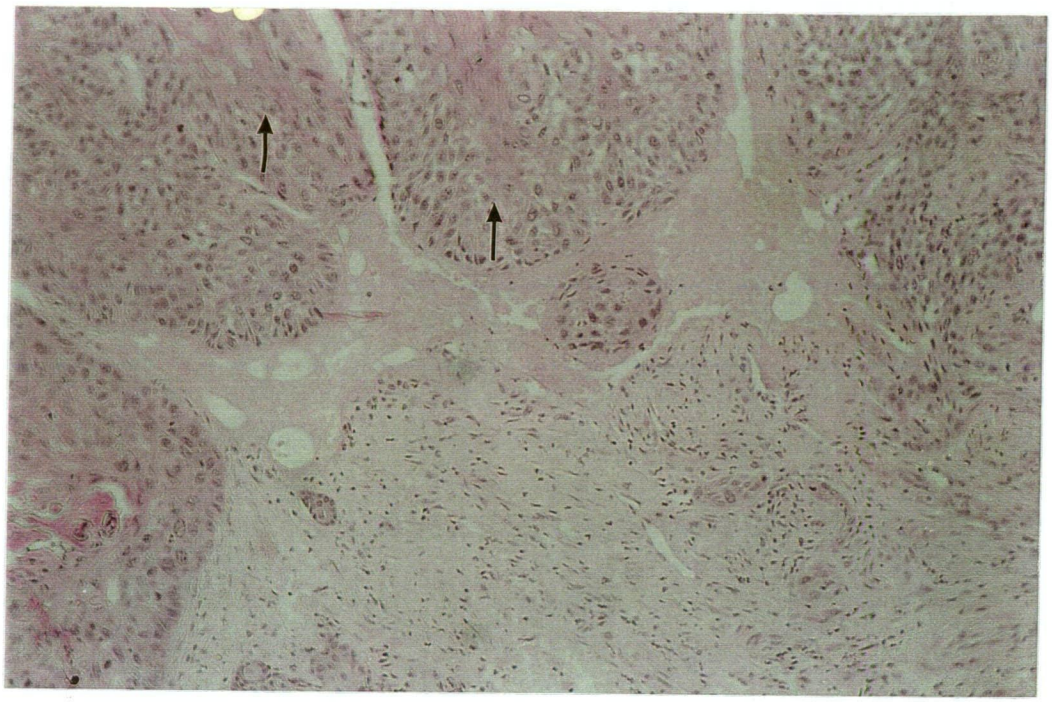

Figure 5 b: Degree of tumour necrosis (moderate).

→ = Necrotic tumour cells

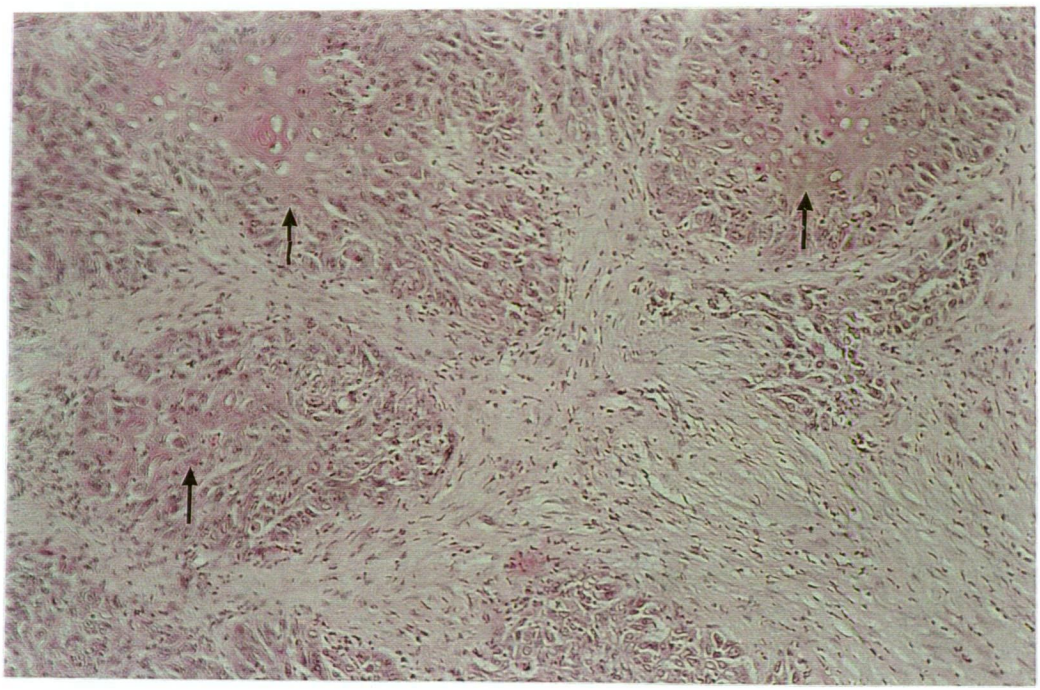

Figure 5 c: Degree of tumour necrosis (marked).

⟶ = Necrotic tumour cells

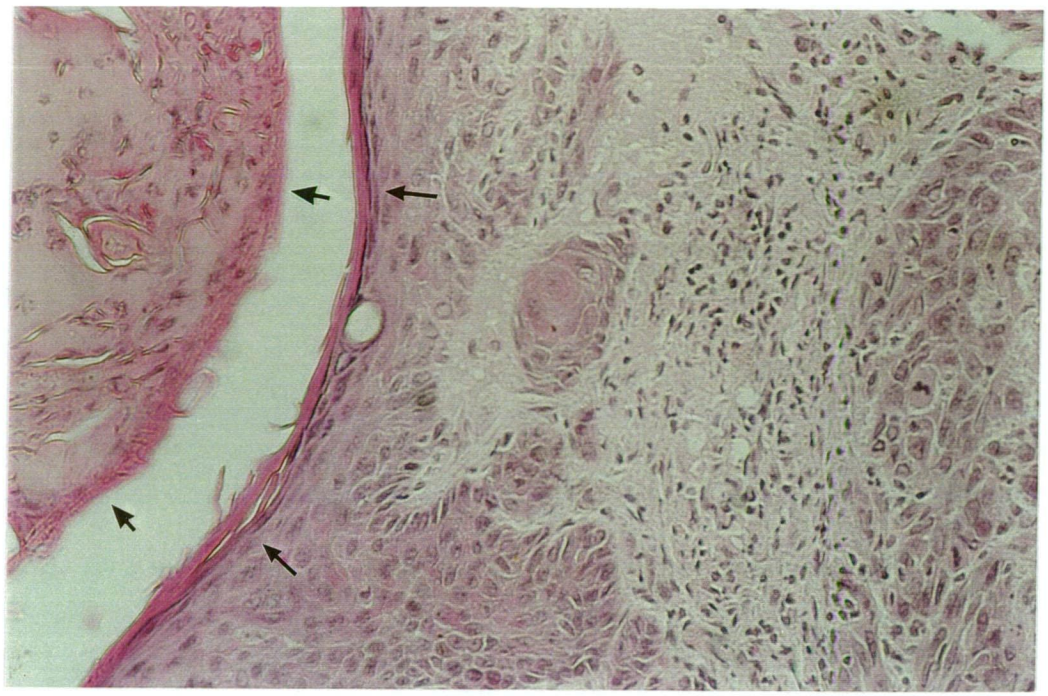

Figure 6: Tumour sloughing and re-epithelization.

Tumour necrosis and sloughing followed by re-epithelization was noticed in some mice in the test group.

➔ = Tumour sloughing

⟶ = Re-epithelization

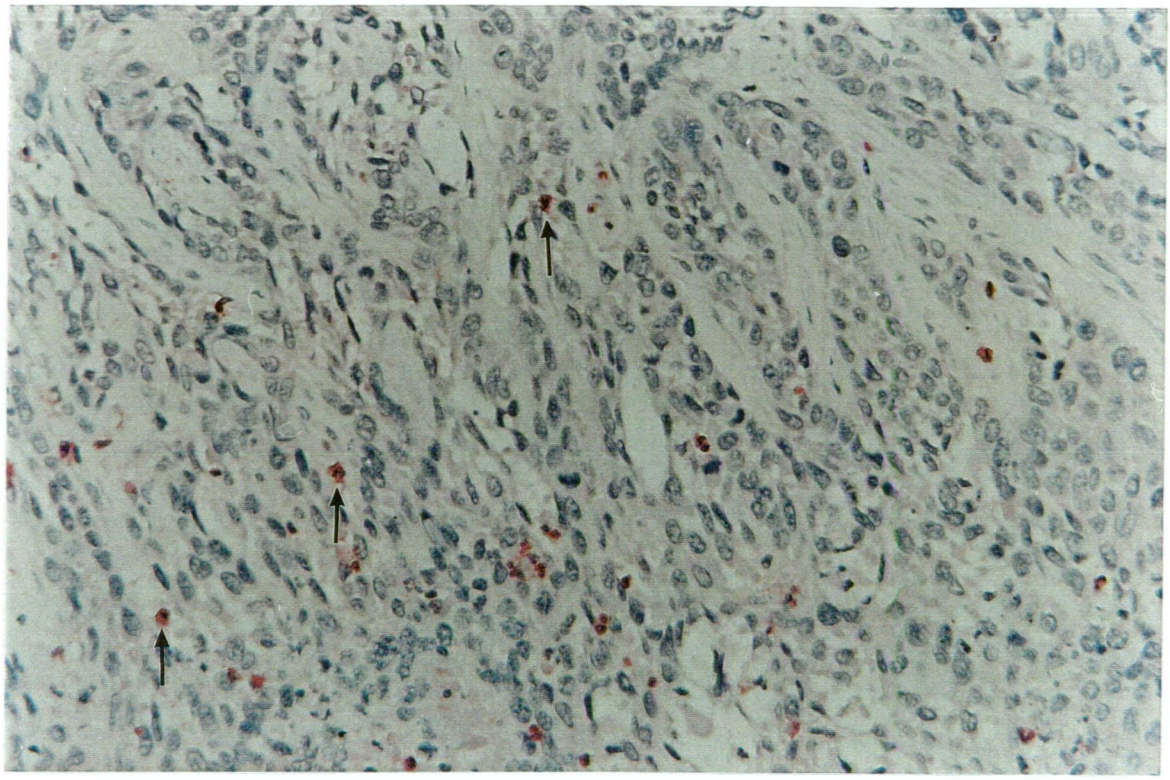

Figure 7: Dendritic cell infiltration of tumours.

→ = Dendritic cells

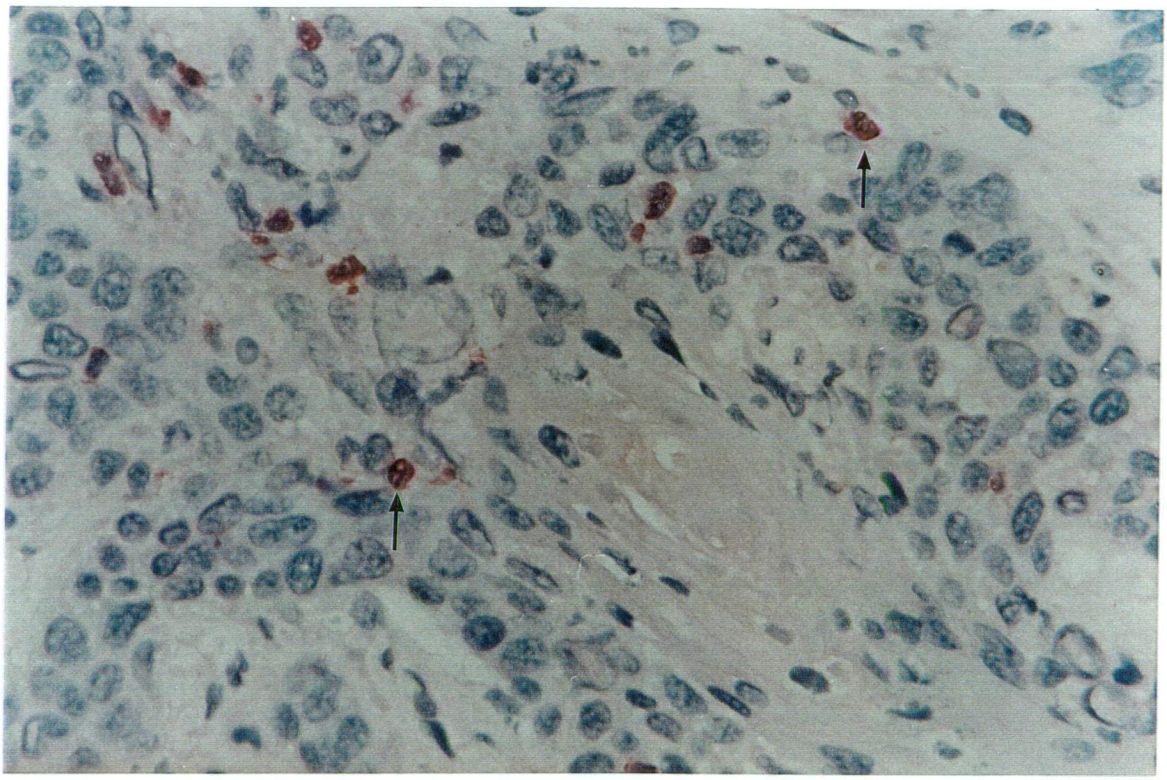

Figure 8: Tumour infiltrating Dendritic cells are round with loss of dendrites.

→ = Dendritic cells

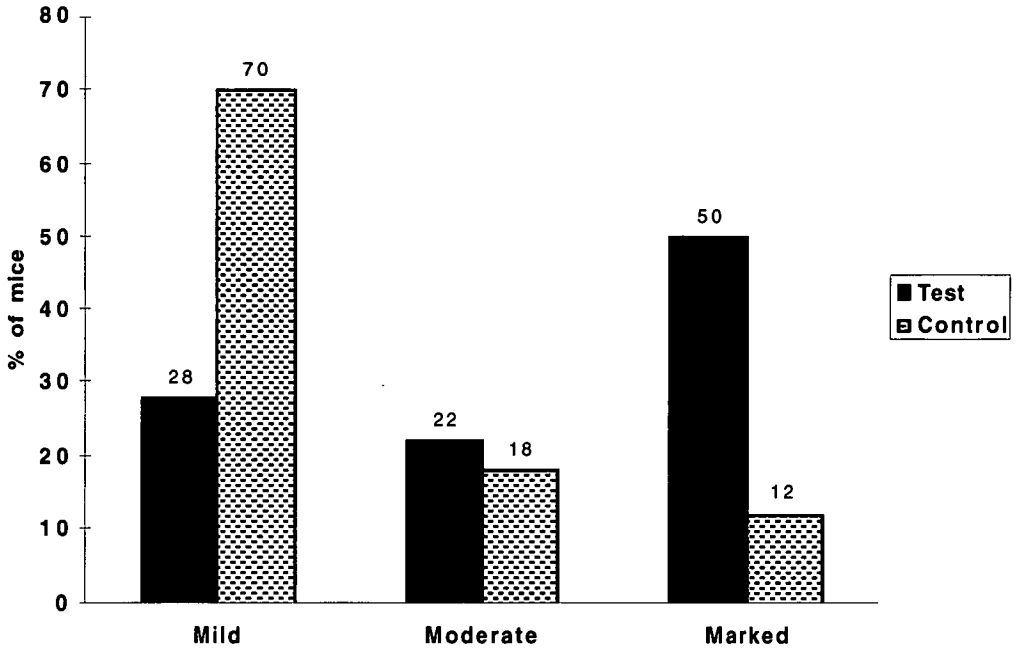

Figure 9: Degree of tumour infiltration by leukocytes was categorised as mild, moderate and marked. Test and control groups were divided into three groups according to the degree of leukocyte infiltration. The results shown are the percentage of mice in each group with comparison between test (n = 17) and control (n = 17) groups.

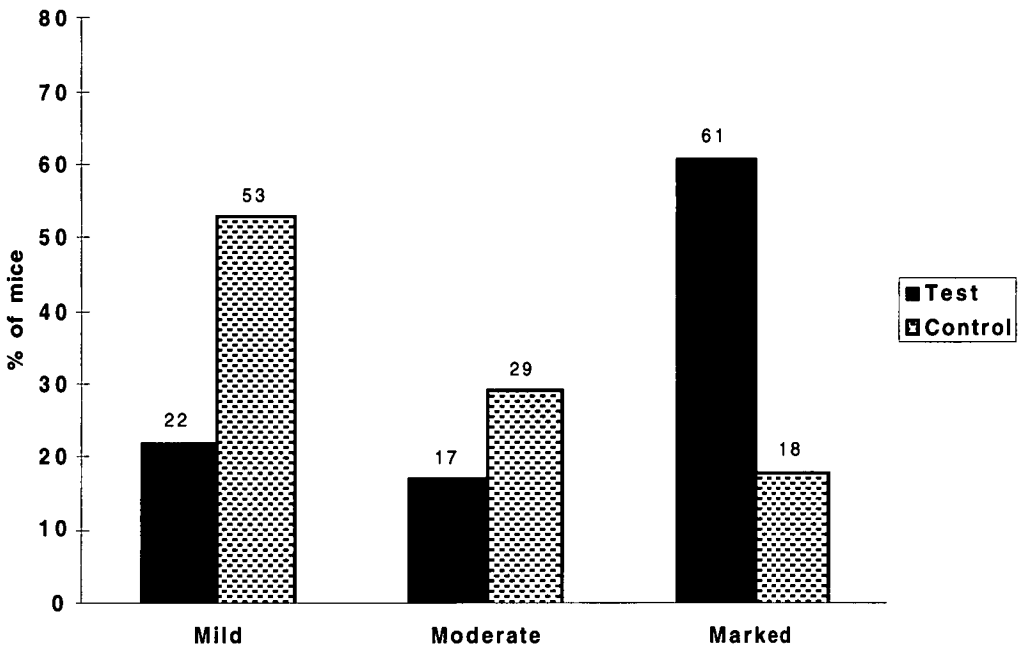

Figure 10: Degree of tumour necrosis was categorised as mild, moderate and marked. Test and control groups were divided into three groups according to the degree of tumour necrosis. The results shown are the percentage of mice in each group with comparison between test (n = 17) and control (n = 17) groups.

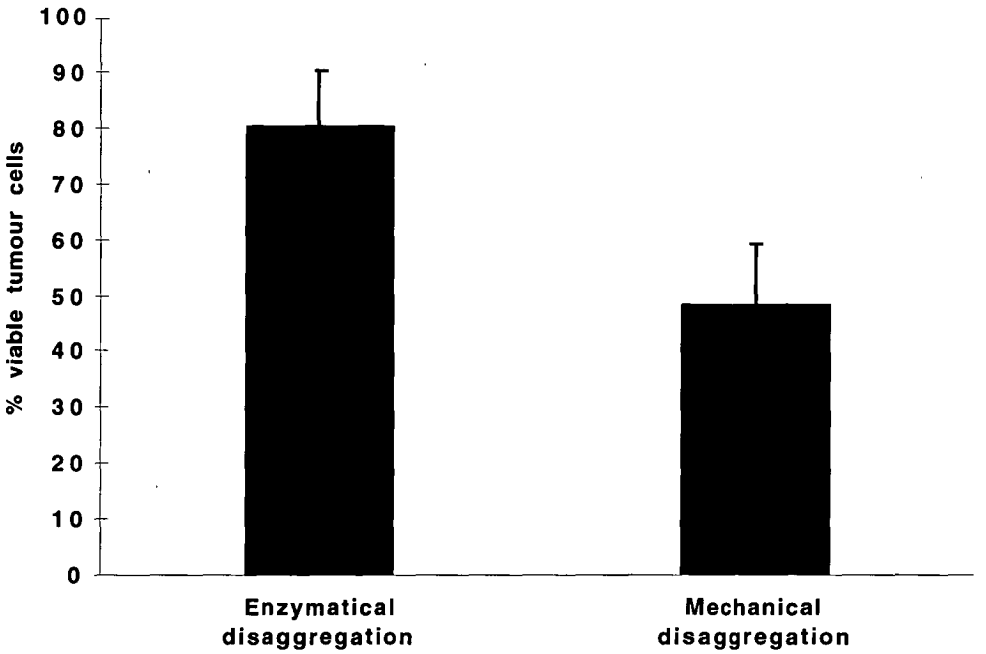

Figure 11: Mechanical and enzymatic disaggregation of tumour cells (mean ± sd).

Mean percentage of viable cells for mechanical disaggregation (48.6 ± 10.6) and for enzymatic disaggregation (80.7 ± 10.1). The difference between the means was significant ($P < 0.05$, student's two-tailed unpaired t-test, $n = 10$)

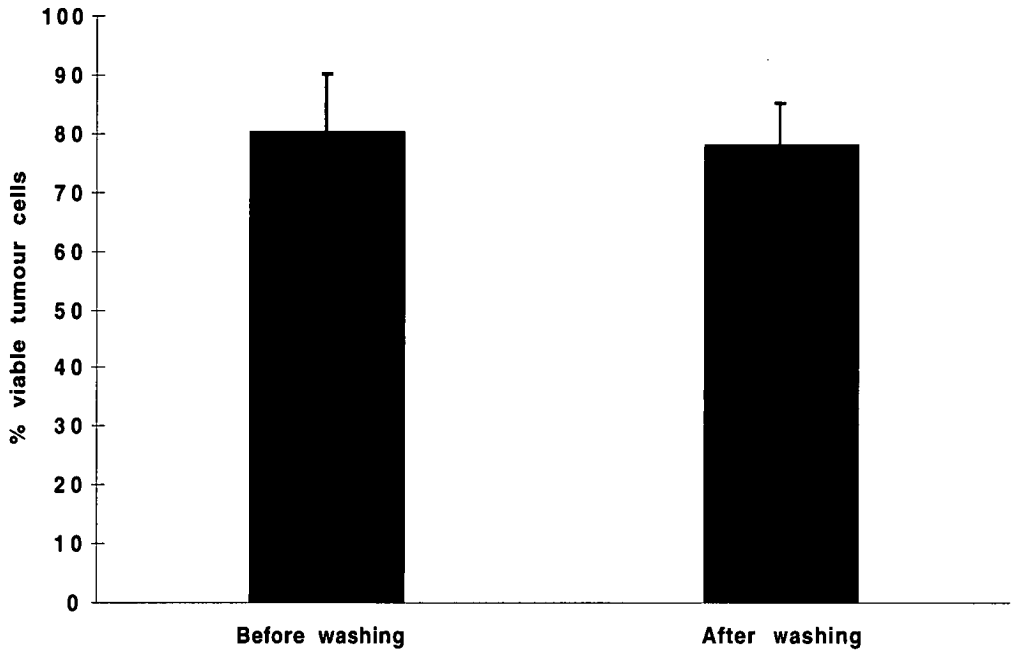

Figure 12: Viability of tumour cells before and after washing (mean ± sd).

Mean percentage of viable cells before washing (80.7 ± 10.1) and after washing (78.2 ± 7.)3. The difference between the means was not significant ($P > 0.05$, student's two-tailed unpaired t-test, $n = 10$).

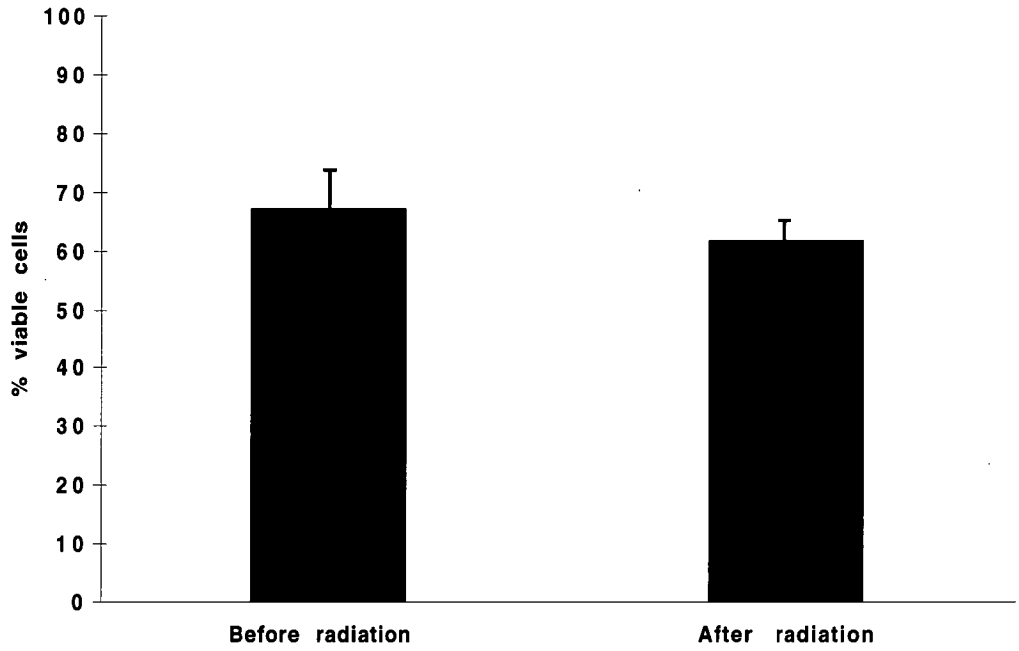

Figure 13: Viability of tumour cells before and after radiation (mean ± sd).

Mean percentage of viable cells before radiation (67.4 ± 6.9) and after radiation (61.6 ± 3.8). The difference between the means was not significant ($P > 0.05$, student's two-tailed unpaired t-test, $n = 24$).

CHAPTER 4

DISCUSSION

The effect of autologous tumour cells in inducing an immune response against tumour cells has been reported in many studies (Currie *et al* 1971; Scharfe *et al* 1986; Slingluff *et al* 1992; Chang *et al* 1997; Berd *et al* 1997; Mackenson *et al* 1997). These observations suggest that TSA are not fully exposed to the immune system in the tumour bearing host, so that introducing tumour cells which carry effective TSA could allow presentation of TSA to the immune system and the development of a host anti-tumour response.

The effect of active specific immunotherapy in this study was evaluated by the degree of tumour infiltration by DC and other immune cells, increased inflammatory infiltration being taken as an indication of the degree of immune response against tumour cells. This assumption is supported by many studies which have shown that tumours which undergo spontaneous regression and those tumours with a better prognosis are infiltrated by DC, T-lymphocytes and other immune cells (Black *et al* 1953, 1958; Tsakraklides *et al* 1974; Bulkley 1975; Morton *et al* 1992; Si *et al* 1996; Berd *et al* 1997), whilst in advanced tumours poor infiltration of tumour by immune cells indicates failure of the immune system (Hakanson *et al* 1997).

The effect of tumour vaccine in this study was evaluated by comparing DC infiltration of tumour tissues in test and control groups. The DC average per 10 HPF (magnification x200) ranged from 0.3 to 24.4 with the mean of 10.5 ± 6.5 in test group and 3.9 ± 2.9 in the control group, the difference in DC infiltration between the two groups being significant ($P < 0.05$), indicating effectiveness of vaccination in inducing or enhancing immune responsiveness to the tumour according to this criterion.

In this study the difference in tumour infiltration by non-DC immune cells between the vaccinated and non-vaccinated groups was significant. In 70% of the control group infiltration was mild compared to 23.5% in the test group. Fifty three percent of the mice in the test group had marked infiltration of their tumour compared to 12% of the control group. In both groups the percentage of mice having moderate infiltration was low (23.5% of the mice in test group and 18% in the control group). The difference between the two groups was significant ($P < 0.05$). Furthermore, there was correlation between tumour infiltration by non-DC leukocytes and tumour infiltration by DC. Tumours with marked infiltration by non-DC leukocytes had the highest DC infiltration (mean 11.1 ± 7.0), whereas tumours with moderate and mild infiltration by non-DC leukocytes had mean DC infiltration of 7.4 ± 5.5 and 4.5 ± 3.7 respectively. The difference between the three groups was significant ($P < 0.05$).This experiment also compared the degree of tumour necrosis between test and control groups. Marked necrosis was detected in 65% of the treated mice compared to 18% of the control. On the

other hand the degree of necrosis in the control group was mild in 53% of the mice while only 17.5% of the test group had mild necrosis. The number of mice which had moderate necrosis was 29% in the control group and 17.5% in the test group. The difference between the control and the treated groups was significant ($P< 0.05$). The area of necrosis was usually surrounded by infiltrating immune cells (lymphocytes, macrophages and neutrophils). There was correlation between tumour necrosis and tumour infiltration by DC. Tumours with marked necrosis had the highest DC infiltration (mean 9.8 ± 5.4), whilst tumours with moderate and mild necrosis had mean DC infiltration of 7.5 ± 7.8 and 4.1 ± 3.8 respectively. The difference between the three groups was significant ($P < 0.05$).

Dendritic cells are highly efficient antigen presenting cells able to process and present antigen to T-cells and to initiate cellular immune response (Stingle *et al* 1978; Sertl *et al* 1986; Hopkins *et al* 1989; Grabbe *et al* 1991, 1992; Van-Schooten *et al* 1997). Since presentation of antigen to the immune system by DC is critical to elicitation of strong immune responses this study evaluated the effectiveness of tumour vaccine on promoting DC infiltration of tumour tissues. Some carcinogens promote tumour development by inhibiting the function or the number of DC, with cessation of carcinogen administration followed by reversible changes of DC number and function and subsequent regression of tumours (Muller *et al* 1985; Zeid *et al* 1995).

Many studies have reported evidence of correlation between the presence

of DC in tumours and good prognosis (Furukwa *et al* 1985; Nomori *et al* 1986; Schroder *et al* 1988; Ambe *et al* 1989; Tsujitani *et al* 1987,1990). The presence of these cells near the tumour is associated with reduced development of metastasis, whilst the absence of these cells is associated with widespread dissemination of tumour cells. Furthermore DC density correlate with tumour histological stage and vascular invasion (Matsuda *et al* 1990; Maehara *et al* 1997). Antigen presentation by DC therefore appears to be a critical component of development of adoptive anti-tumour immunity, this immunity being antigen specific and capable of rejecting tumour cells carrying that antigen (Celluzzi *et al* 1996).

In animal models, regression of metastatic nodules has been observed in tumour-bearing animals treated with DC alone or DC pulsed with tumour extract. DC were able to elicit tumour specific CTL responses in tumour-bearing animals (Yang *et al* 1997b). DC Sensitized to tumour peptide were able to suppress growth of weakly immunogenic tumours, this effect of DC mediated through CTL (Zitvogel *et al* 1996a). In human trials, vaccine consisting of Granulocyte Macrophage-Colony Stimulating Factor (GMCSF) and irradiated autologous melanoma cells led to increased numbers of DC at the vaccination site which was followed by development of Delayed-Type Hypersensitivity (DTH) to autologous tumour cells (Ellem *et al* 1997).

Infiltration of tumour tissue by immune cells other than DC, especially T-cells has also been claimed to be an indication of an effective immune response

against tumour cells. Patients may have better or worse prognosis depending on the degree of tumour infiltration by immune cells, high levels of infiltration being associated with better prognosis, and low or no infiltration with a poor prognosis (Hamlin 1968; Svenning *et al* 1979; Eccles *et al* 1974, Zeid *et al* 1993; Berd *et al* 1997).

Recognition of TSA by DC should be followed by presentation of TSA to T-cells which initiate an immune response against tumour cells carrying this antigen, mediated mainly by CTL (Grabbe *et al* 1991; Bakker *et al* 1995; Zitvogel *et al* 1996; Ellem *et al* 1997; Van-Schooten *et al* 1997; Mayordomo *et al* 1997). Infiltration of tumour tissues by DC therefore may indicate host immunity against tumour cells (Matsuda *et* al 1990; Si *et al* 1996; Maehara *et al* 1997). DC infiltration of tumour thus may serve as an indicator of tumour cell ability to evoke an immune response, i.e. DC could be used as an indicator for the possible presence of TSA which are capable of inducing effective immunity if TSA were to be presented adequately in the presence of a competent immune system. Infiltration of tumour by DC might be used to identify those tumours which are able to evoke an immune response without necessarily having to identify the structure of TSA. Tobacco derived BP-induced tumour has been reported to be highly infiltrated by DC (Ruby *et al* 1989). Regression of tumour induced by tobacco smoke condensate (TSC) and Dimethylbenz-a-anthracene (DMBA) is associated with increased density of DC in these tumours (Muller *et al* 1985; Zeid *et al* 1995). These observations were taken into consideration in this study

in choosing to apply tumour vaccine to BP-induced tumours, since they also tend to be infiltrated by high numbers of DC which might reflect the immunogenicity of these tumours, and provide an opportunity to correlate host responsiveness to extent of DC-infiltration of tumour used for vaccine production. However the ability of some tumours to attract higher numbers of DC than others may partly be linked to the ability of some tumour cells to produce tumour derived cytokines, which attract DC to the lesion (Halliday *et al* 1992). Some tumours with low DC infiltration may express antigens or produce other factors which inhibit DC accumulation and subsequently inhibit tumour immunity (Qin *et al* 1997).

Some immunogenic tumours fail to induce an effective immune response. This may be due to inadequate exposure of TSA to the DC or due to a suppressed immune system, which may recognize the antigen but be unable to produce an effective immune response against that antigen. Activation of T-cells and generation of CTL not only requires processing of TSA by DC in the presence of MHC antigen but also delivery of a costimulatory signal, this signal delivered to CTL through the B7 surface molecule present on the surface of DC. Blocking of this signal or failure of DC to express this molecule leads to inhibition of immune responses (Linsley *et al* 1997; Boussiotis *et al* 1997; Chaux *et al* 1997; Brossart *et al* 1997).

Some studies have reported that DC infiltration of skin tumours is unlikely to reflect the immunogenicity of tumour cells or host immunocompetence

since DC infiltration of immunogenic and non-immunogenic tumours was the same whether these tumours were transplanted into immunocompetent or athymic mice (Bergfelt *et al* 1988; Halliday *et al* 1991). However several recent studies have shown that DC play an important role in immune responses to tumour. Tumour regression is associated with increased numbers of DC (Muller *et al* 1985; Zeid *et al* 1995; Si *et al* 1996), increased numbers of DC are accompanied by immunological and clinical responses, and DC infiltration is decreased in relation to extent of lymph node metastasis (Maehara *et al* 1997). Furthermore the efficacy of DC as a vaccine and the ability of DC to elicit tumour specific CTL have been shown in many recent studies (Mayordomo *et al* 1997; Ellem *et al* 1997; Bronte *et al* 1997; Nair *et al* 1997).

Increased immune cell infiltration of tumours in the test group compared to the control group was taken in this study to reflect enhancement of the immune system by the vaccine. Infiltrating immune cells consisted of lymphocytes, macrophages and neutrophils. However lymphocytes comprised the majority of infiltrating cells. Infiltration by other immune cells such as macrophages and NK cells are also considered part of the immune defence against cancer (Marina *et al* 1981; Trinchieri 1989, Galligioni *et al* 1993; Yang *et al* 1997b; Ellem *et al* 1997). The presence of necrosis within tumour tissues might attract high number of macrophages and neutrophils to the tumour site but this does not exclude the role of these inflammatory cells in an immune response. Some studies showed that healthy tumours and necrotic tumours are infiltrated to the same degree by

macrophages (Evans 1972).

In this study there was no correlation found between the degree of tumour necrosis and the difference of tumour size before and after vaccination. Some mice with marked necrosis had enlarged tumours whilst others with the same degree of necrosis had stable tumour size. Despite the significant difference between the control and the treated mice in the degree of necrosis, no significant difference could be detected in the size of the tumour between both groups. This observation may be due to the fact that the mice were killed three weeks after the first vaccination and this period might not be long enough for clinical regression. Different amounts of tumour volume may also be accounted for early in an anti-tumour immune response by oedema and inflammatory infiltration.

There was no correlation between the degree of DC infiltration of tumours in treated mice and the dose of the vaccine. This indicates that tumour cell dose was effective within the range of 1.53 - 3. 63 X 10^5, however no dose-response conclusions can be drawn. Further experiments with tumour dose beyond these ranges would be required. These results indicate that this vaccine and method of vaccination does induce an immune response to this relatively high DC containing tumour in mice.

The injection site of vaccination was chosen to be away from the tumour site to prevent a direct effect of the vaccine on the tumour tissues. This approach

assured that the effect of the vaccine on the tumour tissues was due to a systemic response to the vaccine, and not a local inflammatory response.

Administration of irradiated tumour vaccine has been shown to lead to lymphocytosis in regional lymph nodes and development of inflammatory response in metastatic tumours (Sensi *et al* 1997). Recent advances in understanding the requirement for T-cell activation indicates that effective antigen presentation is a key requirement for production of tumour specific CTL. TSA should be appropriately processed and displayed in association with MHC antigen (Mackenson *et al* 1997; Guo *et al* 1997; Yang *et al* 1997b). Furthermore a costimulatory signal should be delivered to T-cells though through a molecule on the DC surface to induce an effective immune response (Wang *et al* 1997; Yang *et al* 1997b; Gerstmayer *et al* 1997).

In this experiment part of the tumour was excised surgically to prepare the vaccine. Surgical debulking of tumours may help in producing an immune response against unresected parts of the tumour. The immune response against tumour tissues may be present in the host but are "swamped" by the large amount of antigen present or inhibited by large amounts of immuno-suppressive factors released from the tumour. Partial resection of the tumour may reduce these factors and allow an effective immune response to the residual lesion. In this experiment both groups, control and test, underwent partial resection of their tumours to exclude the possibility that the immune system was enhanced by

reducing tumour mass rather than by the effect of the vaccine.

In the present work animals were exposed to TSA by injecting them with whole irradiated tumour cells. Some trials have used cell fragments as a vaccine presuming the presence of TSA on the cell fragments (Neildhart *et al* 1988; Ravindranath *et al* 1997). Synthetic antigen has also been used to induce immunity against cancer cells carrying that antigen (Blume *et al* 1994; Zhai *et al* 1997). Identification of TSA is a difficult task. TSA have been identified in some histological types of tumours but not others. However identification of TSA does not necessarily mean that the identified antigen is responsible for specific tumour immunity, since tumour cells may carry many tumour antigens, some immunogenic and some not immunogenic (Jager *et al* 1996). Furthermore, more than one antigen may be responsible for specific immune responses against tumour cells and it is difficult to identify the structures of all tumour antigens.

Taking into account these considerations, this study was designed to use irradiated whole tumour cells as active specific immunotherapy. Whole tumour cells are relatively easy to obtain from tumour tissues, and these cells carry TSA whether they are immunogenic or non-immunogenic, so it is not necessary to identify TSA or to identify which TSA are immunogenic and which are not in order to study the immunological response..

In this study autologous tumour vaccine was prepared from squamous cell

carcinomas induced in mice by BP. Autologous cells were used to prepare the vaccine rather than the allogeneic cells from mice of the same strain, as each induced tumour may develop specific TSA different from TSA carried by other tumours of the same histological type and same method of tumour induction (Slingluff 1996). These antigens can provoke an immune response specifically against tumour cells that carry only that antigen (Chang *et al* 1997). Studies which supported the presence of a unique antigen for each tumour despite histological similarity depended on the observation that cross reactivity between allogeneic tumour cells and immune cells did not occur for some tumours. In addition, diversity of tumour antigen is reported in tumours induced by the same carcinogen in different hosts (Klein *et al* 1960; Globerson *et al* 1964; Schirrmacher *et al* 1979; Cortes *et al* 1984). Furthermore recent studies have shown that cytotoxic activity of stimulated PBL against autologous tumour cells are significantly higher than that against allogeneic tumour cells (Strohlein *et al* 1997). The presence of common antigens among tumours of the same histological type has been reported in some tumours however (Morton *et al* 1968; Cornain *et al* 1975; Wagner *et al* 1997). Many studies reported promising results using allogeneic tumour cells as tumour immunotherapy, but this cannot be generalized to all tumour types (Slingluff *et al* 1992; Morton *et al* 1992 ; Yang *et al* 1997a). Allogeneic tumour cells may induce immunity by involving antigens other than TSA on allogeneic tumour cells. In addition identical MHC on tumour cells may be required for an effective immune reaction (Yang *et al* 1997b).

Tumour cells were irradiated before vaccination to stop cell proliferation whilst maintaining the antigenicity of tumour cells. The viability of tumour cells was determined before and after irradiation. The mean percentage of viable cells before irradiation was 67.4% and after irradiation was 61.6%. It has been reported that viability of 33% or more of tumour cells is effective as an immunogenic vaccine (Ahlert *et al* 1997). The difference between the two viability readings in the present study was not significant ($P > 0.05$). This indicates that irradiation did not have significant effect on the viability of tumour cells.

X-ray dosage was adequate to prevent tumour-cell division since no evidence of tumour cell implantation was detected within three weeks after the first vaccination. However this period may not be long enough for clinical evidence of tumour implantation at the site of injection. To exclude the possibility of tumour implantation, tumour cells were incubated for one week in tissue culture medium in a CO_2 incubator after irradiation, whilst non-irradiated tumour cells were incubated as a control for the same period. No significant cell proliferation was detected in flasks containing irradiated cells compared to tumour cell proliferation in flasks containing non-irradiated cells.

Several studies have used cell extract as a vaccine (Neildhart *et al* 1988; Mitchell *et al* 1988; Slingluff *et al* 1992; Ravindranath *et al* 1997). Procedures for preparing cell extract may lead to modification or destruction of tumour antigen which in turn might be responsible for apparent immunity against tumour cells. In

addition recognition of TSA by the immune system requires other cell membrane structures on the tumour cell particularly MHC antigen (Yang *et al* 1997b; Chang *et al* 1997). By using irradiated tumour cells the likelood of TSA alteration or MHC antigen loss was reduced and was more likely to produce a true tumour specific response.

Cryopreservation caused a decrease in cell viability of 10-30%. This drop may have been due to the condition of tumour cells before freezing, since some cells could have been exhausted during disaggregation despite the fact that they appeared viable before freezing. However other factors may affect the viability, such as the technique of thawing tumour cells, the temperature at which the tumour cells are stored and the method of cryopreservation.

Enzymes used for tissue disaggregation may affect the antigens carried by the cells. Some enzymes may directly affect surface antigens leading to decreases in immunogenicity, whilst other enzymes may enhance immunogenicity, for example by removing blocking material or antibodies (Pincus 1981; Seigler *et al* 1979). In this experiment trypsin was used for disaggregation of tumour cells. The effect of this enzyme in modifying TSA could not be excluded since the structure of the TSA responsible for specific tumour immunity in this experiment has not been identified.

In conclusion the data presented in this study has demonstrated that active

specific immunotherapy is able to induce immunity against BP induced squamous cell carcinomas in mice. This tumour type has a relatively high number of DC. Taken together with other studies of tumour types with varying characteristic levels of DC infiltration used to produce vaccines, this study offers supporting evidence that tumours with higher numbers of DC tend to be more responsive to vaccine-induced immunity than tumours with lower numbers of DC. It is possible that DC infiltration could be used as a marker of tumours for which vaccine production is worthwhile. Furthermore, this study has examined DC infiltration as a marker of tumour response to vaccination and provides evidence that DC infiltration does reflect immune response to vaccination, correlating with appearance of tumour necrosis and appearance of non-DC leukocytes.

Further investigation to additionally examine these conclusions could include an attempt to correlate the effectiveness of vaccination with DC infiltration variation within individual tumour types. Additional studies need to be carried out to evaluate the effect of tumour vaccine produced from different histological types of tumours which are characteristically infiltrated by high or low numbers of DC.

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